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Bioremediation of pharmaceuticals by aquatic microorganisms for the recovery of estuarine environments

Joana Pereira Fernandes

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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BIOREMEDIATION OF PHARMACEUTICALS BY AQUATIC MICROORGANISMS FOR THE RECOVERY OF ESTUARINE ENVIRONMENT

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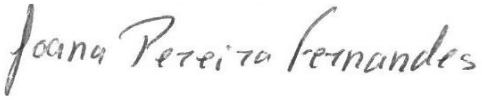
Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico".

Joana Pereira Fernandes



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Abstract

Autochthonous bioremediation has been considered a promising technology for the recovery of different contaminated environments. This technology relies on the key role of native microorganisms for the degradation of pollutants. Therefore, this work aimed to explore the potential of native microorganisms to biodegrade two pharmaceuticals (bezafibrate and paroxetine), for the development of clean-up technologies to recover estuarine environments.

Bacterial strains were isolated from cultures enriched with paroxetine and bezafibrate, using, as microbial inocula, an estuarine sediment and activated sludge from an associated wastewater treatment plant. Bacterial isolates were identified and re-assembled into five consortia, to assess their degradation potential. Three of the five consortia displayed removal efficiencies higher than 97% for paroxetine and bezafibrate. In total, 48 bacterial strains were isolated, being *Pseudomonas* the most abundant genus in all consortia. Two consortia pre-enriched with estuarine sediments, paroxetine degrading consortium and bezafibrate degrading consortium, were selected for further optimization, as they both displayed higher removals.

For the consortia optimization, the potential of each bacterial strain to degrade the respective pharmaceutical was evaluated in biodegradation experiments, either as a single strain or as a consortium, in the presence of a secondary carbon source. Results of paroxetine biodegradation experiments showed that bacteria from the genera *Pseudomonas* and *Leadbetterella* and from the family Chitinophagaceae were the best degrading strains, with removal efficiencies between 81% – 99% and defluorination values between 64% – 77%. As for bezafibrate, bacteria from the genera *Dyadobacter, Leucobacter*, *Microbacterium* and *Ochrobactrum* were the best degrading strains, with removal efficiencies ranging between 67% – 78%. Both consortia displayed a decrease on the performance comparing with the previous experiment, and additional experiments showed that cryopreservation and reactivation process may had a relevant influence on the observed removal efficiencies. In addition, the versatility of each bacterial consortium to bioremediate the other pharmaceutical was also evaluated: paroxetine degrading consortium was able to degrade up to 92% of bezafibrate, and bezafibrate degrading consortium displayed removal efficiency of ca. 85% for paroxetine, in which around 45% of the molecule was defluorinated.

Afterwards, experiments in natural media, were conducted in microcosms with natural estuarine water and sediment, to evaluate the potential of the selected microorganisms to degrade paroxetine, as well as the effects of bioaugmentation process

on the dynamics of estuarine natural community. For this experiment, paroxetine degrading consortium and two bacterial strains, *Pseudomonas* sp. and *Acinetobacter* sp. were selected as bioaugmentation inocula. Paroxetine removals observed in natural media were lower than those obtained in synthetic media, with removal efficiencies up to 50% in water and up to 70% in the presence of sediments, but with a significant contribution of abiotic removal. Low levels of nutrients were observed at the end of the experiment, indicating that nutrients from natural environment were not enough to stimulate the biodegradation process, affecting the removal efficiency. Moreover, the natural microbial community was significantly affected by the presence of paroxetine but not by the addition of the native bacterial strains. The obtained results show that the use of native microorganisms to remove paroxetine in natural environments should be complemented by with the addition of nutrients to stimulate biodegradation activity and fully assess autochthonous bioaugmentation potential.

The present work highlighted the potential of native bacterial strains for the removal of paroxetine and bezafibrate, an important feature for the development of a bioremediation solution to remove paroxetine and bezafibrate from contaminated estuarine environments.

Keywords: bioremediation, autochthonous microorganisms, paroxetine, bezafibrate, estuarine environments.

Resumo

A biorremediação autóctone tem sido considerada uma tecnologia promissora para a recuperação de diferentes ambientes contaminados. Esta tecnologia conta com o papel fundamental dos microrganismos nativos para a degradação de poluentes. Assim, este trabalho teve como objetivo explorar o potencial de microrganismos nativos para biodegradar dois fármacos (bezafibrato e paroxetina), de modo a desenvolver uma tecnologia de remediação capaz de remover estes compostos de ambientes estuarinos contaminados.

Inicialmente, foi realizado o isolamento de diferentes bactérias a partir de culturas enriquecidas com paroxetina e bezafibrato, usando, como inóculo microbiano, um sedimento estuarino e lamas ativadas de uma estação de tratamento de águas residuais (ETAR), que descarrega para o mesmo estuário. Os isolados bacterianos foram identificados e reagrupados adequadamente em cinco consórcios, de forma a avaliar o seu potencial de degradação. Três dos cinco consórcios exibiram eficiências de remoção superiores a 97% para paroxetina e bezafibrato. No total, foram isoladas 48 estirpes bacterianas, sendo o género *Pseudomonas* o mais abundante em todos os consórcios. Dois consórcios pré-enriquecidos com sedimentos estuarinos, denominados consórcio degradador da paroxetina e consórcio degradador do bezafibrato, foram selecionados para otimização posterior, uma vez que ambos apresentaram remoções elevadas.

Para a otimização dos consórcios, o potencial de cada estirpe bacteriana para degradar o respetivo fármaco foi avaliado em experiências de biodegradação, em cultura pura ou em consórcio, na presença de fonte secundária de carbono. Os resultados das experiências de biodegradação da paroxetina mostraram que as bactérias dos géneros *Pseudomonas* e *Leadbetterella* e da família Chitinophagaceae foram as que apresentaram melhores capacidades de degradação, com eficiências de remoção entre 81% – 99% e valores de defluorinação entre 64% – 77%. Quanto ao bezafibrato, as bactérias dos géneros *Dyadobacter*, *Leucobacter*, *Microbacterium* e *Ochrobactrum* foram as que apresentaram melhores capacidades de degradação, com eficiências de remoção variando entre 67% – 78%. Ambos os consórcios apresentaram uma diminuição no desempenho em comparação com a experiência anterior, e experiências adicionais mostraram que o processo de criopreservação e reativação pode ter uma influência relevante nas eficiências de remoção observadas. Além disso, a versatilidade de cada consórcio bacteriano para biorremediação do outro fármaco foi também avaliada: o consórcio degradador da paroxetina foi capaz de degradar até 92% do bezafibrato, e o consórcio degradador do

bezafibrato exibiu eficiência de remoção de *ca*. 85% para a paroxetina, na qual cerca de 45% da molécula foi defluorinada.

Posteriormente, foram realizadas experiências em meio natural para avaliar o potencial dos microrganismos para degradar paroxetina bem como os efeitos do bioaumento na dinâmica da comunidade. Para esta experiência foram selecionados o consórcio degradador da paroxetina e dois isolados, *Pseudomona*s DPS 10 e *Acinetobacte*r DPS 5. As remoções de paroxetina observadas em meio natural foram inferiores às obtidas em meio sintético, com eficiências de remoção até 50% na água e até 70% na presença de sedimentos, mas com contribuição significativa da remoção abiótica. Níveis baixos de nutrientes foram observados no final da experiência, indicando que os nutrientes do ambiente natural não foram suficientes para estimular o processo de biodegradação, afetando a eficiência de remoção. Além disso, a comunidade microbiana natural foi significativamente afetada pela presença de paroxetina, mas não pela adição das estirpes bacterianas nativas. Os resultados obtidos mostram que o uso de microrganismos nativos para remover a paroxetina em ambientes naturais deve ser complementado com a adição de nutrientes para estimular a atividade de biodegradação e avaliar plenamente o potencial do bioaumento autóctone.

O presente trabalho destacou o potencial de isolados bacterianos autóctones para a remoção de paroxetina e bezafibrato, um fator importante para o desenvolvimento da tecnologia de biorremediação, para remover estes fármacos de ambientes estuarinos contaminados.

Palavras chave: biorremediação, microrganismos autóctones, paroxetina, bezafibrato, ambientes estuarinos

Index

Chapter 1 - Introduction 31
Recovering pharmaceutical-contaminated ecosystems through bioremediation – a review....31
1.1 Introduction 33
1.2 Pharmaceutical compounds and their environmental deleterious effects....36
1.3 Pharmaceuticals in the environment....37
1.4 Therapeutic classes of pharmaceuticals: environmental presence and effects....40
1.4.1 Antibiotics 40
1.4.2 Nonsteroidal Anti-inflammatory drugs....42
1.4.3 Antidepressants 43
1.4.4 Blood lipid lowering agents....46
1.5 Analytical methods for detection of pharmaceuticals in the environment 48
1.6 Removal of pharmaceuticals in wastewater treatment plants and factors that can affect their removal 49
1.7 Bioremediation processes as a new remediation technology....53
1.7.1 Biodegradation of pharmaceuticals by single strains 54
1.7.2 Biodegradation by bacterial consortia....56
1.7.3 Factors affecting biodegradation process....57
1.8 General overview 59
1.9 Aims and general structure of the PhD Thesis 61
Chapter 2....63
Potential of bacterial consortia obtained from different environments for bioremediation of paroxetine and bezafibrate 63
2.1 Abstract....65
2.2 Introduction 66
2.3 Material and methods....68
2.3.1 Reagents 68
2.3.2 Isolation of bacterial strains from cultures enriched with paroxetine and bezafibrate 69
2.3.3 Taxonomic identification of the purified bacterial strains....71
2.3.4 Biodegradation of paroxetine and bezafibrate by bacterial consortia assembled with isolated strains....72
2.3.5 Analytical methods....72

2.3.5.1 Analysis of the concentration of paroxetine and bezafibrate in the culture medium 72
2.3.5.2 Analysis of fluoride ion release 73
2.3.6 Statistical analysis 73
2.4 Results 73
2.4.1 Bacterial composition of the consortia 73
2.4.2 Biodegradation experiments 83
2.5 Discussion 85
2.6 Conclusions 90
Chapter 3 93
Potential of native bacterial strains from estuarine environments to degrade halogenated pharmaceuticals 93
3.1 Abstract 95
3.2 Introduction 96
3.3. Material and Methods 99
3.3.1. Reagents 99
3.3.2. Optimization of bacterial consortia 99
3.3.3. Optimization of a paroxetine degrading consortium (DPS) 99
3.3.3.1. Biodegradation experiments with single bacterial strains 99
3.3.3.2. Biodegradation experiments for the optimization of DPS consortium 101
3.3.3.3. Bacterial growth with different carbon sources 101
3.3.3.4. Biodegradation experiments starting with different optical density 102
3.3.3.5. Bacterial growth with different reactivation periods for Chitinophagaceae family DPS 4 102
3.3.3.6. Biodegradation experiments for DPS consortium with different reactivation period 102
3.3.4. Optimization of a bezafibrate degrading (DBS) consortium 102
3.3.4.1. Bacterial growth with different carbon sources 103
3.3.4.2. Biodegradation experiments with single bacterial strains 103
3.3.4.3. Biodegradation experiments starting with different optical density 103
3.3.4.4. Bacterial consortia with different pharmaceuticals 103
3.3.5. Analytical methods 104
3.3.5.1. Pharmaceuticals determination 104
3.3.5.2. Fluoride ion release 104
3.3.6. Statistical analysis 105

3.4 Results 105
3.4.1. Biodegradation by bacterial consortia and isolated bacterial strains 105
3.4.1.1 Paroxetine 105
3.4.1.1.1. Biodegradation experiments with single bacterial strains 105
3.4.1.1.2 Biodegradation experiments for the optimization of a DPS consortium 110
3.4.1.1.3 Bacterial growth with different carbon sources 111
3.4.1.1.4 Biodegradation experiments starting with different optical density 114
3.4.1.1.5 Bacterial growth with different reactivation periods for Chitinophagaceae family DPS 4 115
3.4.1.1.6 Biodegradation experiments for DPS consortium with different reactivation periods 115
3.4.1.2 Bezafibrate 116
3.4.1.2.1. Bacterial growth with different carbon sources 116
3.4.1.2.2 Biodegradation experiments with single bacterial strains 118
3.4.1.2.3 Biodegradation experiments starting with different optical density 122
3.4.2. Biodegradation by bacterial consortia and isolated bacterial strains 122
3.5 Discussion 124
3.6 Conclusions 130
Chapter 4 133
Autochthonous bioaugmentation for bioremediation of paroxetine in natural media – microbial community dynamic and biodegradation potential 133
4.1 Abstract 135
4.2 Introduction 136
4.3 Methodology 138
4.3.1 Reagents and materials 138
4.3.2 Sampling 138
4.3.3 Biodegradation experiments in natural media 139
4.3.4 Isolation of bioaugmented bacterial strains and taxonomic identification by sanger sequencing 16S rRNA gene 141
4.3.5 Microbial community structure 142
4.3.6 Paroxetine determination 143
4.3.6.1 Water samples 143
4.3.6.2 Sediment samples 144
4.3.6.3 Nutrient Analysis 144

4.3.7 Data Analysis 144
4.3.7.1 Microbial community analysis 144
4.3.7.2 Multinomial Species Classification Method (CLAM) 145
4.3.7.3 NGS dataset *vs* isolated bioaugmented strains....146
4.3.7.4 Pharmaceuticals removal and nutrient levels....147
4.4 Results....147
4.4.1 Microbial Community Dynamics 147
4.4.2 Alpha Diversity....147
4.4.3 Microbial community structure - Beta diversity 148
4.4.4 Characterization of the prokaryotic communities 150
4.4.5 Recovered bacterial strains by culture dependent and independent methods....153
4.4.6 Generalists Versus Specialists: CLAM analysis 157
4.4.7 Analytical Analysis 159
4.4.7.1 Nutrient consumption....159
4.4.7.2 Paroxetine determination....161
4.4.7.2.1 Microcosm with and without sediment....161
4.5 Discussion....163
4.6 Conclusions 169
Chapter 5....171
General Discussion and Conclusions....171
5.1 General discussion....173
5.2 Conclusions 181
Chapter 6....183
References 183
Appendix....229
Appendix I....231
Appendix II....263

Figures Index

Figure 1 - Input and environmental fate of pharmaceuticals in the environment and bioremediation process as a solution. C – carbon; N – nitrogen; P – phosphorous 35
Figure 2 – Different removal mechanisms of pharmaceuticals that can occur in the environment. 40
Figure 3 - Experimental design for the isolation and purification of bacterial strains from the enriched consortia and assemblage of biodegradation experiments. Bzf – bezafibrate; Prx – Paroxetine. DPS- estuarine sediment under static conditions, exposed to paroxetine; DBS - estuarine sediment under static conditions, exposed to bezafibrate; EPS- activated sludge under static conditions, exposed to paroxetine; EBS – activated sludge under static conditions, exposed to bezafibrate; EBO – activated sludge under agitated conditions, exposed to bezafibrate ; AS – activated sludge. PCA – plate count Agar; MM – mineral-salts medium.....70
Figure 4 - Relative abundance of bacterial phyla within the different cultures pre-enriched with paroxetine and bezafibrate selected for this study. 74
Figure 5 - Relative abundance in terms of bacterial genera (except for Chitinophagaceae affiliates and Rhodobacteraceae affiliates for which only family was obtained) of the strains isolated from the cultures pre-enriched with paroxetine and bezafibrate selected for this study. 75
Figure 6 - Phylogenetic tree of four potential new species belonging to the phylum Bacteroidetes, isolated from estuarine sediment enriched cultures or activated sludge enriched cultures, based on 16S rRNA gene homology with their nearest strains (26 sequences, with a final alignment with 1394 bp). Numbers at nodes represent bootstrap values when higher than 60%. Numbers in each branch corresponds to GenBank accession numbers. Phylogenetic tree built based on the Maximum Likelihood test, with Hasegawa-Kishino-Yano (HKY + G + I) nucleotide evolutionary model. 76
Figure 7 - Phylogenetic tree of two potential new species belonging to the phylum Proteobacteria, isolated from activated sludge enriched cultures, based on 16S rRNA gene homology with their nearest strains (36 sequences, with a final alignment with 1391 bp). Numbers at nodes represent bootstrap values when higher than 60%. Numbers in each branch correspond to GenBank accession numbers. Phylogenetic tree built based on the Maximum Likelihood test, with Tamura 3 parameter (T92 + G + I) nucleotide evolutionary model.....77
Figure 8 - Phylogenetic tree of one potential new species belonging to the phylum Actinobacteria, isolated from estuarine sediment enriched cultures, based on 16S rRNA

gene homology with their nearest strains (9 sequences, with a final alignment with 1436 bp). Numbers at nodes represent bootstrap values when higher than 60%. Numbers in each branch correspond to GenBank accession numbers. Phylogenetic tree built based on the Maximum Likelihood test, with Kimura 2 parameter (K2 + G + I) nucleotide evolutionary model.....78

Figure 9 - Removal efficiency of paroxetine and bezafibrate by the consortia derived from estuarine sediment and activated sludge, along three feeding cycles. Week 2 – end of 1st cycle, week 4 – end of 2nd cycle, Week 6 – end of 3rd cycle. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences among the same inoculum, at different cycles ($p<0.05$); b - significant differences within the different inocula, for the same pharmaceutical and at the same cycle ($p<0.05$); c – significant differences among the same inoculum, for different incubation conditions ($p<0.05$). 83

Figure 10 - Defluorination of paroxetine by the consortia derived from estuarine sediment and activated sludge, along three feeding cycles. Week 2 – end of 1st cycle, week 4 – end of 2nd cycle, Week 6 – end of 3rd cycle. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences among the same inoculum, at different cycles ($p<0.05$); b - significant differences within the different inocula ($p<0.05$). 84

Figure 11- Schematic overview of the experiments conducted in chapter 2. OD – optical density; DPS – Paroxetine degrading consortium; DBS – Bezafibrate degrading consortium; OPDPS – optimized DPS consortium; PCA – plate count agar 98

Figure 12 - Removal efficiency of paroxetine for each bacterial strain and by the paroxetine degrading consortium (DPS), after four weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a - significant differences among each treatment throughout time ($p<0.05$). 107

Figure 13 - Defluorination of paroxetine by each bacterial strain and by the paroxetine degrading consortium (DPS), after four weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences among each treatment throughout time ($p<0.05$). 108

Figure 14 - Bacterial growth, determined by measurement of optical density at 600nm, of each DPS bacterial strain and DPS consortium, for 4 weeks of experiment. 109

Figure 15 - Defluorination of paroxetine by paroxetine degrading consortium and optimized consortium (OPDPS), for 1 mg L^{-1} and 2 mg L^{-1}, after 4 weeks of experiment. Results are

expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences among treatment for at different concentrations (p<0.05).....110
Figure 16 - Bacterial growth, determined by measurement of optical density at 600nm, for DPS and OPDPS consortia for 4 weeks of experiment. 111
Figure 17 - Bacterial growth, determined by measurement of optical density at 600nm, for each bacterial strain of DPS consortium in Mineral-salt (MM) medium, only in the presence of sodium acetate. 113
Figure 18 - Bacterial growth, determined by measurement of optical density at 600nm, for Bosea DPS2, Chitinophagaceae family DPS 4, Leadbetterella DPS 8 and Microbacterium DPS 9, in the presence of different carbon sources. 114
Figure 19 - Defluorination of paroxetine by paroxetine degrading consortium (DPS) starting with different optical density (OD), after three weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation.....114
Figure 20 - Bacterial growth displayed by the Chitinophagaceae family DPS 4, for different cultivation periods, before inoculation in MM media. PCA 2.5 – Strain cultivated in PCA for 2 weeks and a half; PCA 1 – Strain cultivated in PCA for 1 week.....115
Figure 21 - Defluorination of paroxetine by paroxetine degrading consortium (DPS) after four weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences on removal efficiency throughout time (p<0.05).....116
Figure 22 - Bacterial growth, determined by measurement of optical density at 600nm, for each bacterial strain of DBS consortium in Mineral-salt (MM) medium, only in the presence of sodium acetate. 117
Figure 23 - Bacterial growth, determined by measurement of optical density at 600nm, for Herminiimonas DBS 2, Dyadobacter DBS 3 and Leucobacter DBS 5, in the presence of different carbon sources. 118
Figure 24 - Removal efficiency of bezafibrate by each bacterial strain and by the bezafibrate degrading consortium (DBS), after four weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. DBS_Y – Bezafibrate degrading consortium using Yeats extract as second carbon source. * - Bacterial isolates using Yeats extract as second carbon source. a - significant differences among each treatment throughout time (p<0.05).....120
Figure 25 - Bacterial growth, determined by measurement of optical density at 600nm, of each DBS bacterial strain and DBS consortium, for 4 weeks of experiment. DBS –

bezafibrate degrading consortium; DBS_Y – bezafibrate degrading consortium feed with yeast extract * - bacterial strains supplemented with yeast extract.....121
Figure 26 - Removal bezafibrate by bezafibrate degrading consortium (DBS) starting with different optical density (OD), after three weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a - significant differences among each treatment throughout time ($p<0.05$); b - significant differences in each treatment at different ODs at the same time ($p<0.05$).....122
Figure 27 - Removal efficiency and defluorination of paroxetine performed by the bezafibrate degrading consortium, after 4 weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a - significant differences on removal efficiency throughout time ($p<0.05$). b - significant differences on defluorination throughout time ($p<0.05$) 123
Figure 28 - Removal efficiency of bezafibrate performed by the paroxetine degrading consortium, after 4 weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a - significant differences on removal efficiency throughout time ($p<0.05$).....123
Figure 29 - Bacterial growth, determined by measurement of optical density at 600nm, for DPS (exposed to bezafibrate) and DBS (exposed to paroxetine) consortia for 4 weeks of experiment.....124
Figure 30 - Experimental design of biodegradation experiments in natural media (estuarine water and sediment). Ct - Control; PRX Cte – abiotic control; PRX Ct – control with paroxetine; PRX Ac – microcosms bioaugmented with Acinetobacter DPS 5; PRX Ps – microcosms bioaugmented with Pseudomonas DPS 10; PRX Mx, - Microcosms bioaugmented with DPS consortium. All microcosms with the PRX stands for microcosms exposed to paroxetine.....140
Figure 31 - Alpha diversity (observed OTUs and Shannon diversity) in the initial sediments and sediments from each treatment, after two weeks of experiment. IS – Initial sediment; Ct -Control (natural community), PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with Acinetobacter DPS 5; PRX Ps – Bioaugmented with Pseudomonas DPS 10; PRX Mx– Bioaugmented with bacterial consortium (DPS). 148
Figure 32 - Beta diversity represented by Non-metric multidimensional scaling ordination based on Bray–Curtis dissimilarities of bacterial communities in initial sediments and in each treatment after two weeks of experiment. IS – Initial sediment; Ct -Control (natural community), PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with Acinetobacter

DPS 5; PRX Ps – Bioaugmented with Pseudomonas DPS 10; PRX Mx– Bioaugmented with bacterial consortium (DPS). 149
Figure 33 - Taxonomic profile at phylum level in the initial sediment and in sediments for each treatment, after 2 weeks of experiment. IS – Initial sediment; Ct -Control (natural community), PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with Acinetobacter DPS 5; PRX Ps – Bioaugmented with Pseudomonas DPS 10; PRX Mx– Bioaugmented with bacterial consortium (DPS). 151
Figure 34 - Taxonomic profile at genus level in the initial sediment and in sediments after 2 weeks of each treatment after two weeks of experiment. IS – Initial sediment; Ct -Control (natural community), PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with Acinetobacter DPS 5; PRX Ps – Bioaugmented with Pseudomonas DPS10; PRX Mx– Bioaugmented with bacterial consortium (DPS). 152
Figure 35 - Heatmap representing the presence of bioaugmented strains in each treatment, using NGS data set against the consensus sequences of the bioaugmented strains. 157
Figure 36 - Prokaryotic community classified as specialists by Multinomial Species Classification Method (CLAM) analysis in the control without paroxetine (Experimental control, Ct). 158
Figure 37 - Prokaryotic community classified as specialists by Multinomial Species Classification Method (CLAM) analysis in the control with paroxetine (PRX Ct). 158
Figure 38 - Characterization of water regarding the nutrients concentrations (phosphate, ammonium, nitrite and nitrate ions (mean and standard deviation, n = 3) in the different treatments, for the microcosms without sediment. a - Significant differences among the initial water (IW) and sterile control (PRX Cte); b – Significant differences among the sterile control (PRX Cte) and biotic treatments at the same time ($p < 0.05$); c – significant differences among the control with paroxetine and bioaugmented treatments ($p<0.05$). IW – Initial water; PRX Cte - sterile control; PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with Acinetobacter DPS 5; PRX Ps – Bioaugmented with Pseudomonas DPS 10; PRX Mx– Bioaugmented with bacterial consortium. 160
Figure 39 - Characterization of water regarding the nutrients concentrations (phosphate, ammonium, nitrite and nitrate ions (mean and standard deviation, n = 3) in the different treatments, for the microcosms with sediment. a - Significant differences among the initial water and sterile control; b – Significant differences among the sterile control (abiotic) and biotic treatments at the same time ($p < 0.05$); c – significant differences among the control with paroxetine and bioaugmented treatments ($p<0.05$). IS – Initial sediment; PRX Cte - sterile control; PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with

Acinetobacter DPS 5; PRX Ps – Bioaugmented with Pseudomonas DPS 10; PRX Mx– Bioaugmented with bacterial consortium.....161
Figure 40 - Removal efficiency of paroxetine in the microcosms with and without sediment, in each treatment, after two weeks of experiment. Removal for microcosms with sediment were calculated taking into consideration the paroxetine detected in the sediments and water samples. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences among the sterile control (abiotic) and biotic treatments ($p < 0.05$). b - significant differences among the control (PRX Ct) and bioaugmented treatments ($p < 0.05$); c – significant differences among bioaugmented treatments. PRX Cte - sterile control; PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with Acinetobacter DPS 5; PRX Ps – Bioaugmented with Pseudomonas DPS 10; PRX Mx– Bioaugmented with bacterial consortium.....162

Tables Index

Table 1 - Taxonomic identification of bacterial isolates obtained from the cultures pre-enriched with paroxetine, using estuarine sediment (Douro river estuary) and activated sludge from an associated WWTP as inocula. Strains isolated from estuarine sediment under static incubation were named as DPS, while isolates retrieved from activated sludge, also under static incubation, were named as EPS. Bp – base pairs; PCA –Plate Count Agar; MM - Mineral-salts Medium. * potential new species according to the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database. 79

Table 2 - Taxonomic identification of bacterial isolates obtained from the cultures pre-enriched with bezafibrate using estuarine sediment (Douro river estuary) and activated sludge from an associated WWTP as inocula. Strains isolated from estuarine sediment under static incubation were named as DBS; isolates retrieved from activated sludge, also under static incubation, were named as EBS and isolates obtained from activated sludge under agitation incubation were named as EBO. Bp – base pairs; PCA –Plate Count Agar; MM - Mineral-salts Medium. * potential new species according to the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database. 81

Table 3 - Taxonomic identification of bacterial isolates from paroxetine degrading consortium (DPS) and bezafibrate degrading consortium (DBS), used in the biodegradation experiments, as a consortium or as a single strain. Adapted from Fernandes et al. (2020). MM - Mineral-salt agar; PCA – plate count agar; Prx – paroxetine; Bzf - Bezafibrate.....100

Table 4 - Alignment scores between Paroxetine degrading strains and Bezafibrate degrading strains, among the same genera. 119

Table 5 - Taxonomic identification of bacterial isolates used in the biodegradation experiments. PCA –Plate Count Agar; MM - Mineral-salts Medium. PRX Ac – microcosms bioaugmented with Acinetobacter DPS 5, PRX Ps – microcosms bioaugmented with Pseudomonas DPS 10, PRX Mx, - Microcosms bioaugmented with DPS consortium Adapted from Fernandes et al. (2020), chapter 2. 141

Table 6 - One-way ANOSIM analysis for the experimental conditions, bioaugmentation and pharmaceutical effect on the prokaryotic community structure (9999 permutations). 150

Table 7 - Bacterial isolates recovered from microcosms without sediment. n.a – not applied. PRX Ac – Bioaugmented with Acinetobacter DPS 5; PRX Ps – Bioaugmented with Pseudomonas DPS 10; PRX Mx– Bioaugmented with bacterial consortium (DPS).....155

Table 8 - Bacterial isolates recovered from microcosms assembled with sediment. n.a – not applied. PRX Ac – Bioaugmented with Acinetobacter DPS 5; PRX Ps – Bioaugmented with Pseudomonas DPS 10; PRX Mx– Bioaugmented with bacterial consortium (DPS).....156

Supplementary Tables Index

Table S 1 - Pharmaceuticals concentration detected in freshwater samples (expressed in ng L^{-1}). 232

Table S 2 - Pharmaceuticals concentration detected in underground water samples (expressed in ng L^{-1}). 240

Table S 3 - Pharmaceuticals concentration detected in seawater samples (expressed in ng L^{-1}). 243

Table S 4 - Pharmaceuticals concentration detected in drinking water samples (expressed in ng L^{-1}). 244

Table S 5 - Pharmaceuticals concentration detected in sediment and soils samples (expressed in ng g^{-1}). 247

Table S 6 - Pharmaceuticals concentration detected in sludge samples (expressed in ng g^{-1}). 251

Table S 7 - Pharmaceuticals concentration detected in wastewaters effluent samples (expressed in ng L^{-1}). 254

List of Abbreviations

AIC - Akaike Information Criterion

AMO - Ammonium Monooxygenase Enzyme

AOPs - Advanced Oxidation Processes

BAC - Biological Activated Carbon

BLAST - Basic Local Alignment Search Tool

Bp – Base pairs

CAS - Conventional Activated Sludge

CECs - Contaminants of Emerging Concern

COVID-19 - Coronavirus SARS-CoV-2

COX-1 - Cyclooxygenase Isoform 1

COX-2 - Cyclooxygenase Isoform 2

DDD - Defined Daily Doses

DGGE - Denaturing Gradient Gel Electrophoresis

ECDC - European Centre for Disease Prevention and Control

F/M - Food to Microorganisms

g L^{-1} – Gram per Liter

GAC - Granular Activated Carbon

GC-MS/MS - Gas chromatography - Tandem Mass Spectrometry

h - Hours

HCl – Hydrochloric Acid

HKY + G + I - Hasegawa-Kishino-Yano

HMG-CoA - 3-hydroxy-3-methylglutaryl Coenzyme A

HPLC - High-Performance Liquid Chromatography

HPLC-DAD (Diode Array Detector)

HQ - Hazard Quotients

HRMS - High-Resolution Mass Spectrometry

HRT - Hydraulic Retention Time

I3S - Instituto de Investigação e Inovação em Saúde

IC10 - 10% Inhibitory Concentration

INFARMED - Autoridade Nacional do Medicamento e Produtos de Saúde, I.P.

K2 + G + I - Kimura 2 Parameter

KH - Henry Law Constant

Koc - Soil Adsorption Coefficient

Kow - Octanol-Water Partition Coefficient

LC-MS/MS - Liquid Chromatography - Tandem Mass Spectrometry

LLE - Liquid-Liquid Extraction

LLME - Liquid–Liquid Membrane Extraction

MAOIs - Monoamine Oxidase Inhibitors

MBR - Membrane Bioreactor

MEGA - Molecular Evolutionary Genetics Analysis

mg L^{-1} – Milligram per Liter

MGEs - Mobile Genetic Elements

min - Minutes

mM – Millimolar

Mm – Micrometre

MM - Mineral-Salts Medium

mRNA – messenger Ribonucleic Acid

NCBI - National Center for Biotechnology Information

ng g^{-1} – Nanogram per Gram

ng L^{-1} – Nanogram per Liter

NGS – Next Generation Sequencing

nm – Nanometres

NSAIDs - Nonsteroidal Anti-Inflammatory Drugs

°C – Celsius Degree

OD - Optical Density

OECD - Organization for Economic Cooperation and Development

OTUs - Operational Taxonomic Units

PAC - Powdered Activated Carbon

PCA - Plate Count Agar

PCR - Polymerase Chain Reaction

pKa - Negative base-10 logarithm of the Acid Dissociation Constant (Ka)

PPCPs - Pharmaceutical and Personal Care Products

PSI – Photosystem I

PSII – Photosystem II

QqLIT-MS/MS - Hybrid Triple Quadrupole-Linear Ion Trap Mass Spectrometer

RDP - Ribosomal Database Project

Rpm - Revolutions per Minute

RQ - Risk Quotient

s - Seconds

SNRIs - Norepinephrine Reuptake Inhibitors

SPE - Solid Phase Extraction

SPME - Solid Phase Microextraction

SRT - Sludge Retention Time

SSRIs - Selective Serotonin Reuptake Inhibitors

T92 + G + I - Tamura 3 Parameter

TCAs - Tricyclic Antidepressants

TISAB - Total Ionic Strength Adjustment Buffer

UASB - Upflow Anaerobic Sludge Blanket Reactor

UHPLC - Ultra-High-Performance Liquid Chromatography

UK – United Kingdom

USA – United States of America

UV - Ultraviolet

v/v – Volume per Volume

WWTPs - Wastewater Treatment Plants

λ – Wavelength

µL - Microliter

µg L^{-1} -Microgram per Liter

µM - Micromolar

16S rRNA gene - 16S ribosomal Ribonucleic acid

Chapter 1 - Introduction

Recovering pharmaceutical-contaminated ecosystems through bioremediation – a review

1.1 Introduction

In the last years, the presence of the so-called contaminants of emerging concern (CECs) in the environment has been reported worldwide, mostly due to the development of industrial and medicinal sectors. Although, the presence of CECs has been occurring for decades in the environment, only in the past 10-15 years analytical methods with the capacity to detect these pollutants at environmentally relevant concentrations were developed (Onesios et al., 2009; Ternes, 1998).

CECs comprise different contaminants, such as pharmaceuticals, engineered nanomaterials, illicit drugs, synthetic musks, food additives, phthalates, artificial sweeteners, hormones, steroids, industrial compounds/by-products, personal care and veterinary products (Gavrilescu et al., 2015; Lapworth et al., 2012; Mailler et al., 2016; Thomaidis et al., 2013). For these compounds, no current regulations on water quality framework exist despite their potential threat to the environment and public health (Ncibi et al., 2017; Thomaidis et al., 2013).

Until recently, monitoring or public reports of the presence of CECs in freshwaters or wastewaters were not required (Silva et al., 2013). In 2013, the Directive 2013/39/EU (The European Parlament and the Council of the European Union, 2013) was implemented as an amendment to the Directives 2000/60/EC and 2008/105/EC, encompassing CECs as priority substances in the field of water policy to minimize aquatic environmental contamination by these compounds. To achieve this goal, new high-quality monitoring and prioritization measures were implemented, according to the article 16 of Directive 2000/60/EC (The European Parlament and the Council of the European Union, 2000, 2008). A Watch List, containing several contaminants of emerging concern, was created to register the monitoring data and establish the risk that these selected contaminants may have in the environment (Commission Implementing Decision (EU) 2015/495, 2015). The first Watch List was published by the Decision 2015/495/EU (Commission Implementing Decision (EU) 2015/495, 2015) and revised by the Decision 2018/840/EU (Commission Implementing Decision (EU) 2018/840, 2018). In 2020, a new revised version was published, in the Decision 2020/1161/EU, including 18 CECs that should be monitored to gather information for further evaluation (Commission Implementing Decision (EU) 2020/1161, 2020).

In the last years, the risks associated with the presence of CECs in the environment and public health have been attracting more attention. Many CECs can be toxic, persistent and non-biodegradable (Wang & Wang, 2018), being their natural removal more difficult. However, even the non-persistent compounds can cause negative effects in the aquatic systems and organisms (Rivera-Jaimes et al., 2018) if their continuous input in the

environment overcomes their natural attenuation and transformation (Petrović et al., 2003; Quesada et al., 2019). Therefore, besides monitoring and controlling CECs emissions, their removal from the impacted environments is necessary.

Pharmaceuticals, a well-known class of CECs, have been essential for the sustainability and maintenance of human health, ensuring life quality (Caracciolo et al., 2015) and illness relief. Nonetheless, their extensive consumption has led to their presence in the environment, threatening the living organisms (Ebele et al., 2017). The uncertainty regarding their effects on different non target organisms has been raising concern among the scientific community. Thus, there is an urgent need for the development of technologies suitable to be applied in the environment to tackle the contamination by these pollutants.

In this chapter, concerns regarding the presence, occurrence and fate of pharmaceuticals, as well as their potential effects in different environments and in non-target organisms, are addressed. Among the different classes of pharmaceuticals, anti-inflammatory drugs, antidepressants, antibiotics and blood lipid lowering agents were chosen for a more accurate review, as they are among the most prescribed and consumed pharmaceuticals in the world. Autochthonous bioremediation, using native bacteria, raises as a solution for the recovery of contaminated environments, exploring and enhancing the natural biological mechanisms for the removal of pharmaceuticals (Figure 1). Several case studies are presented showing the potential of this technology for further applications.

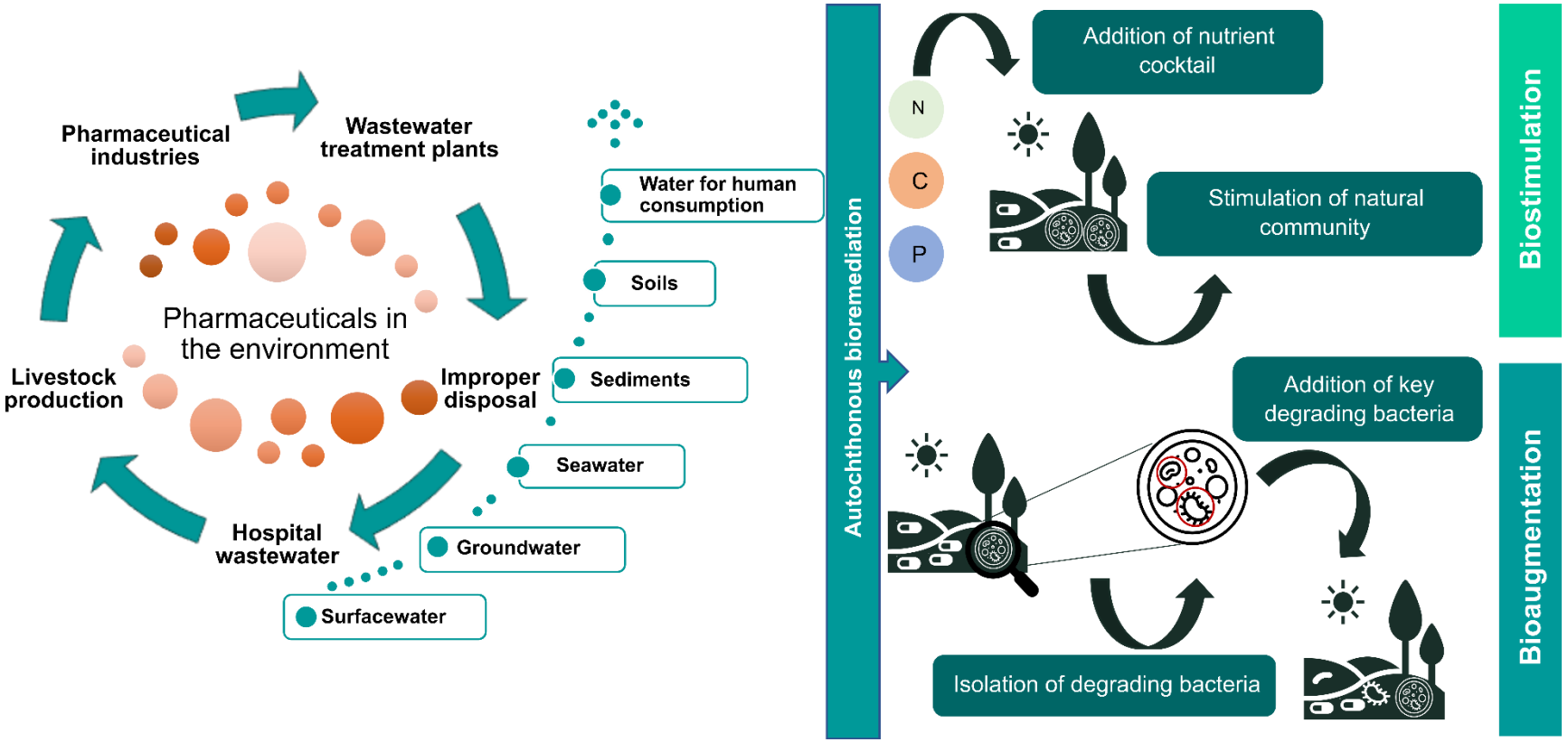

Figure 1 - Input and environmental fate of pharmaceuticals in the environment and bioremediation process as a solution. C – carbon; N – nitrogen; P – phosphorous

1.2 Pharmaceutical compounds and their environmental deleterious effects

Pharmaceuticals are natural or synthetic compounds used in human and veterinary medicine to treat various diseases. These compounds are usually polar molecules, with more than one ionizable group and different structures and functions, tending to be lipophilic or moderately soluble in water (Quesada et al., 2019). Pharmaceuticals have the ability to pass through cellular membranes and to remain as active molecules when excreted to the environment (Quesada et al., 2019 and references therein).

Pharmaceuticals are divided into 24 therapeutic classes, which comprise around 10 000 different pharmaceuticals containing about 3000-4000 different active ingredients (FDA, 2020; Stadlmair et al., 2018). The most consumed ones are antibiotics, anti-inflammatory, analgesics, antidepressants, antiepileptic, lipid-lowering drugs, β-blockers, antiulcer drugs and antihistamines (Guerra et al., 2014; Rivera-Utrilla et al., 2013). The persistence of pharmaceuticals in the environment, together with their extensive and growing use and production, continuous environmental input at low concentrations (ranging from µg L^{-1} to ng L^{-1}) and potential toxicological effects on non-target organisms (Caracciolo et al., 2015; Heiss & Küster, 2015; Puckowski et al., 2016; Silva et al., 2015) have become an issue for the scientific community. In addition, the deleterious effects that these compounds may have on ecosystems functioning and structure and on human health has been also raising concern (Ebele et al., 2017; Onesios et al., 2009; Picó & Andreu, 2007). Experimental studies showed that pharmaceuticals may cause endocrine disruption, change the structure and key functions of natural communities, affect negatively invertebrates and fishes and, in the case of antibiotics, lead to the development of antibiotic resistant genes and bacteria (Alvarino et al., 2014; Ding & He, 2010; Fernandes et al., 2015; Finley et al., 2013; Marti et al., 2014; Pomati et al., 2006; Wellington et al., 2013). As an example, a sublethal dose of mianserin, a tetracyclic antidepressant, promoted a significant inhibition on the growth of zebrafish larvae (*Danio rerio*), altering their physiological and biochemical parameters (Yang et al., 2018). In another study, Guiloski and collaborators reported oxidative stress, inhibition of liver enzymes, genotoxicity and changes in steroid hormones (among others) on *Rhamdia quelen* (catfish), due to paracetamol exposure (Guiloski et al., 2017).

The available data on the environmental presence of pharmaceuticals is unsatisfactory to understand which compounds present the highest threats to the environment. As such, it is necessary and recommended to perform more representative

acute and chronic toxicity tests of pharmaceuticals in a representative range of aquatic organisms. Parolini & Binelli (2012), reported cyto-genotoxic effects on zebra mussels due to a short-term exposure to a nonsteroidal anti-inflammatory drugs (NSAIDs) mixture that caused an increase in oxidative stress, which in turn led to genetic damage (Parolini & Binelli, 2012). The authors also highlighted that the observed cyto-genotoxic damages were higher when NSAIDs was added as a mixture than when a single NSAID compound was administrated (Parolini & Binelli, 2012). In another study, the Hazard Quotients (HQ) for several pharmaceuticals found in Apatlaco River (Mexico) were observed throughout different trophic levels (*daphnia*, algae and fish) (Rivera-Jaimes et al., 2018). The mentioned studied concluded that *daphnia* was more sensitive to bezafibrate, acetaminophen, carbamazepine and naproxen (Rivera-Jaimes et al., 2018). On the other hand, algae displayed more negative effects to sulfamethoxazole, indomethacin and trimethoprim, while atenolol, gemfibrozil, diclofenac, ibuprofen and salicylic acid were the most toxic pharmaceuticals to fish (Rivera-Jaimes et al., 2018). Rivera-Jaimes and collaborators also concluded that the high concentrations of ibuprofen, sulfamethoxazole, diclofenac and naproxen found in the river water might pose a relevant risk to the whole aquatic ecosystem (Rivera-Jaimes et al., 2018).

With the increasing evidence of the negative effects of pharmaceuticals in the environment and aquatic life, more attention has been paid to this issue. Despite the lack of regulation regarding the presence of most pharmaceuticals in aquatic environments, some of these compounds have been already included in the 2nd and 3rd Watch List, as is the case of (i) the antibiotics amoxicillin, ciprofloxacin, erythromycin, clarithromycin, azithromycin, sulfamethoxazole and trimethoprim; (ii) the hormones 17-Alpha-ethinylestradiol, 17-Beta-estradiol, and estrone, (iii) the synthetic hormone norethisterone; (iv) the antidepressant venlafaxine and (v) three antifungal pharmaceuticals, clotrimazole, fluconazole, miconazole (Cortes et al., 2020; Loos et al., 2018).

1.3 Pharmaceuticals in the environment

The presence of pharmaceuticals in the environment has been widely reported in the last decades, being a worldwide issue of increasing concern (Roig & D'Aco, 2016). Development and improvement of analytical techniques allowed the detection of pharmaceuticals at environmental relevant concentrations with more sensitivity and precision.

Mixtures of pharmaceuticals, along with their active metabolites, are being unceasingly introduced into the environment through wastewater and sewage treatment

plants effluents from municipalities, hospitals, livestock and pharmaceutical industries, illegal untreated effluent discharges, improper disposal of unused or expired pharmaceuticals, manufacture spill accidents, manure and sludge use as organic fertilizer, treatment of crop diseases and sometimes through leachates from solid waste landfills (Eslami et al., 2015; Finley et al., 2013; Madureira et al., 2010a, 2010; Silva et al., 2015; Togunde et al., 2012; Wellington et al., 2013). Pharmaceuticals and their metabolites have been reported to occur in surface waters (Table S1), groundwaters (Table S2), coastal marine waters (Table S3), water for human consumption (Table S4), soils and estuarine sediments (Table S5) (Caracciolo et al., 2015; Marti et al., 2014; Paíga et al., 2015). However, very little attention has been paid to the metabolites and transformation products resultant from the metabolism of these compounds (López-Serna et al., 2012). Therefore, monitoring programs should also include the analysis of these molecules as they can be more toxic and persistent than the parent compound and also induce negative effects on aquatic organisms and ecosystems (Ebele et al., 2017; Farré et al., 2008; Noguera-Oviedo & Aga, 2016; Quesada et al., 2019).

One of the main concerns related to the presence of pharmaceuticals in the environment is their usual occurrence as complex mixtures rather than as a single compound, hardening the development of clean up technologies for their removal from the contaminated sites. The various pharmaceuticals that are continuously and simultaneously used and released into the environment can interact synergistically (Calisto & Esteves, 2009; Daughton & Ternes, 1999), affecting non-target organisms, since they can possess similar molecular targets (Silva et al., 2015). In addition, the environmental concentration of pharmaceuticals, as well as their synergistic and antagonist effects are directly related to the geographical area, climatologic conditions and occurrence of wastewater discharges (Santos et al., 2009).

Once in the environment, the persistence of pharmaceutical products can be influenced by (i) environmental factors (pH, soil characteristics, temperature, light incidence), (ii) physicochemical properties of the molecule (solubility (expressed by the octanol-water partition coefficient, (K_{ow})), molecular structure, polarity, pK_a, photo-stability, chemical stability, volatility (expressed by the Henry law constant (K_H)), (iii) presence of other pharmaceuticals in the same matrix (antagonistic and synergistic effects), and (iv) presence and activity of microorganisms with the ability to degrade pharmaceuticals, metabolizing them as carbon sources or as co-metabolites (Caracciolo et al., 2015; Dordio & Carvalho, 2013; Gros et al., 2007; Onesios et al., 2009; Tiwari et al., 2017; Vasquez et al., 2014). The presence of other biodegradable organic carbon sources can improve the

removal/degradation of pharmaceutical compounds by enhancing the growth of degrading microorganisms or co-metabolic processes (Alexandrino et al., 2020; Carvalho et al., 2016; Moreira et al., 2018).

The current concentration of pharmaceuticals released into the environment exceeds its natural capability to degrade them. The natural attenuation and detoxification of pharmaceuticals in the environment can occur through sorption, hydrolysis, photolysis, dispersion, biodegradation, dilution and, more rarely, through radioactive decomposition (Figure 2) (Castro et al., 2018; Kramer et al., 2018; Lahti & Oikari, 2011; Lin et al., 2010; Parezanović et al., 2019; Ziylan & Ince, 2011). Nevertheless, a review on the fate of pharmaceuticals in sewage and freshwaters suggests that hydrolysis might not have a significant role in its elimination from the environment, being photodegradation and biodegradation the processes described to be more relevant in that mechanism (Ziylan & Ince, 2011). As case studies, Lin and collaborators (2010) described biodegradation as a key removal mechanism for acetaminophen, an analgesic and antipyretic drug, in natural aqueous systems, however, for propranolol and acebutolol removal (both used to treat hypertension), sorption was the dominant mechanism (Lin et al., 2010). Fluoxetine, an antidepressant, was reported to be removed from surface waters due to natural depuration by microbial communities, with an estimated half-life of 6 to 10 days (Benotti & Brownawell, 2009; Nödler et al., 2014). However, this effect was not observed by Rúa-Gómez & Püttmann (2013) for the antidepressant venlafaxine, which showed a slow biotic degradation in surface waters (Rúa-Gómez & Püttmann, 2013). Similar results were obtained by Aymerich and collaborators (2016) where venlafaxine did not display natural attenuation (Aymerich et al., 2016). Venlafaxine and fluoxetine displayed different behaviors in natural environments (surface waters). The concentration and chemical structure of the two compounds, as well as the natural microbial community that was subjected to the experiment are features that influence the natural depuration of pharmaceuticals and change the behavior of pharmaceuticals within the same family of compounds.

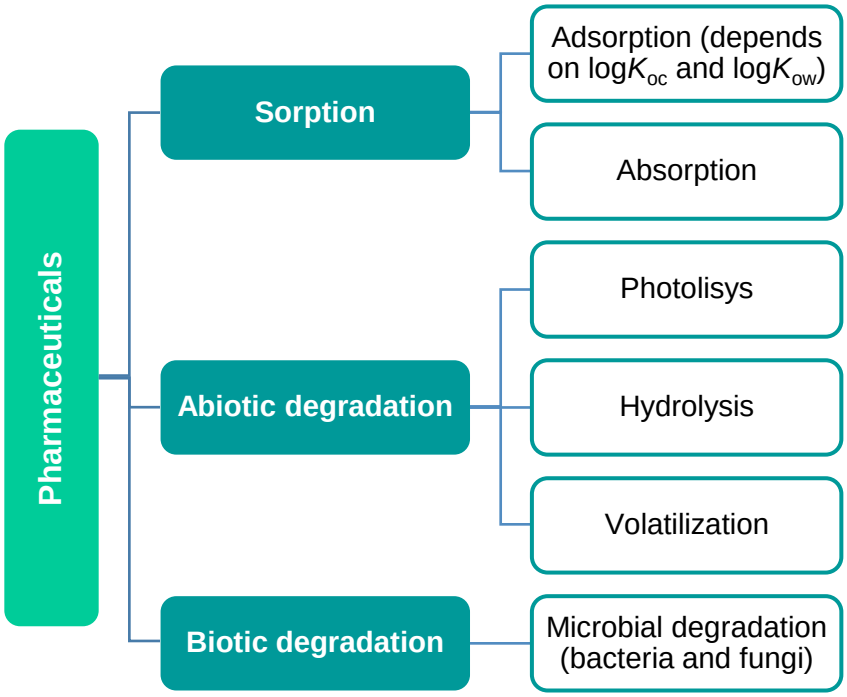

Figure 2 – Different removal mechanisms of pharmaceuticals that can occur in the environment.

1.4 Therapeutic classes of pharmaceuticals: environmental presence and effects

1.4.1 Antibiotics

Antibiotics are one of the most used group of pharmaceuticals in human and veterinary medicine. These pharmaceuticals are divided in several classes, such as quinolones (and fluoroquinolones), tetracyclines, macrolides, sulfonamides and β-Lactams (Ding & He, 2010; Kümmerer, 2009). Fluoroquinolones, macrolides and aminoglycosides are frequently the most prescribed antibiotics in human medicine, while penicillins, tetracyclines and macrolides are the most frequently prescribed antibiotics in veterinary medicine (Milić et al., 2013). Nearly 250 antibiotics are hitherto registered in human and veterinary medicine (Kümmerer, 2009 and references therein). The origin of antibiotics can be natural (usually products from the secondary metabolism of fungi or bacteria), semi-synthetic (byproducts derived from natural products) or synthetic (Caracciolo et al., 2015).

According to the European Centre for Disease Prevention and Control (ECDC) reports, the overall antibiotic consumption in the European Union and European Economic Area (expressed in defined daily doses (DDD) per 1 000 inhabitants and per day), between

2010 and 2014, had a significant increasing trend (ECDC, 2015), but in 2017, the consumption of antibiotics slightly decreased in some countries (Finland, Germany, Italy, Luxembourg, Netherlands, Norway, Sweden and the United Kingdom (UK)). Penicillins, quinolones, cephalosporins and other β-Lactams and macrolides were the antibiotics most consumed between 2013-2017 (ECDC, 2018). In the report of 2019, eleven countries (Austria, Belgium, Finland, Germany, Italy, Luxembourg, Netherlands, Portugal, Slovenia, UK and Sweden) displayed a significant decreasing trend on total antibiotics consumption for systemic use, while Bulgaria, Iceland, Latvia and Ireland displayed a significant increasing trend (ECDC, 2019).

In Portugal, amoxicillin and amoxicillin + clavulanic acid (penicillin), azithromycin (macrolide) and ciprofloxacin (fluoroquinolone) were the antibiotics most consumed between 2012 – 2016 (INFARMED). Also, according to the Organization for Economic Cooperation and Development (OECD) Health Data report of 2015, Portugal registered the 12th highest volume of antibiotics prescribed between 2000 and 2013. On the other hand, Turkey, Greece, France and Italy registered the highest consumption levels (OECD, 2015), a tendency maintained in general in the last years (OECD, 2019).

Antibiotics have been detected in different environmental matrices such as soils, freshwaters, seawater, groundwater and even in water for human consumption, at different concentrations (Tables S1 to S7). For instance, ciprofloxacin, a fluoroquinolone worldwide used, was found in freshwaters at levels ranging between 0.53 and 740 ng L^{-1} (Gracia-Lor et al., 2011; Osorio et al., 2016; Petrović et al., 2014) (Table S1), in groundwater at concentrations ranging between 51 and 443 ng L^{-1} (López-Serna et al., 2013) (Table S2) and at lower concentrations in sediments, ranging between 0.1 and 42 ng g^{-1} (Osorio et al., 2016; Vazquez-Roig et al., 2012; Wu et al., 2014) (Table S5). In addition, concentrations between 2130 and 45590 ng g^{-1} were found in wastewaters sludge, for the same pharmaceutical (Leal et al., 2012; Zhao et al., 2010) (Table S6). For erythromycin, a macrolide antibiotic, concentration values ranging between 0.45 and 1378 ng L^{-1} were found in freshwaters (Kay et al., 2017; Osorio et al., 2016) (Table S1), while in drinking water and groundwaters concentrations of around 5 ng L^{-1} (Gaffney et al., 2015) and 40 ng L^{-1} (López-Serna et al., 2013) respectively, were reported (Tables S2 and S4). This antibiotic was also found in sludge from wastewaters at low concentrations (12 – 32 ng g^{-1}) (Ho et al., 2014) (Table S6).

The presence of antibiotics in the environment and their improper use became an issue of increasing awareness and concern since they promote bacterial resistance. This phenomenon can occur through several and complex mechanisms, namely, via intracellular

modification and/or deactivation of the antibiotic, exclusion of the antibiotic by the cell membrane, intracellular sequestration, reduction of the cellular target sensitivity and extrusion from the cell (Marti et al., 2014 and references therein). Very low or sub-inhibitory antibiotic concentrations, comparable to those found in environmental reservoirs (water matrices and soil), can potentiate the selection of resistant bacteria and the horizontal exchange of mobile genetic elements (MGEs) encoding antibiotic resistance genes (Marti et al., 2014). In addition, antibiotics can decrease denitrification rates, affect methanogenesis and sulfate reduction processes and induce the death and/or inhibit degrading microorganisms in sewage treatment plants, soil and water ecosystems (Caracciolo et al., 2015), which can have deleterious effects in the ecosystems.

1.4.2 Nonsteroidal Anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a very heterogenous group of pharmaceuticals, extensively used all over the world to treat a huge number of common acute and chronic inflammatory processes (Manrique-Moreno et al., 2016; Monteiro et al., 2017). NSAIDs are commonly used to treat symptoms of inflammation and pain, to relieve fever and sometimes, depending on the substance, to treat rheumatic diseases (Embrandiri et al., 2016). NSAIDs are weak acids that act as non-selective inhibitors of one of the cyclooxygenase isoforms, COX-1 and COX-2, two enzymes involved in the biosynthesis of prostaglandins that mediate pathogenic processes, including the inflammatory reaction (Manrique-Moreno et al., 2016; Mezzelani et al., 2016; Ricciotti & FitzGerald, 2011; Santos et al., 2010).

Among the different classes of pharmaceuticals, NSAIDs are one of the most used in therapeutics, not only in terms of prescription but also in terms of self-medication, mainly because of their low prices and over-the-counter accessibility (Elizalde-Velázquez et al., 2020; Manrique-Moreno et al., 2016). The consumption of NSAIDs has been increasing 11.9% each year in UK, United States of America (USA), France, Italy, Spain and Japan; this is the equivalent to approximately 30 million people consuming NSAIDs every day (Feng et al., 2013). In Portugal, more than 6000000 packages of NSAIDs were consumed in 2016, being ibuprofen and diclofenac among the most used NSAIDs (INFARMED).

The intensive consumption of NSAIDs leads to their significant detection in wastewater treatment plant (WWTPs) effluents and consequent release to the aquatic environment due to their inefficient removal in those wastewater treatment plants (Cortés et al., 2013; Ziylan & Ince, 2011). These pharmaceutical compounds are the most frequently detected in the aquatic environment (Cortés et al., 2013), representing 15% of the total

drugs detected in monitoring studies worldwide. Some anti-inflammatory drugs such as ketoprofen, fenoprofen, naproxen, mefenamic acid, diclofenac and ibuprofen, were found in the aquatic environment at μg L^{-1} levels, wherein a significant portion comes from wastewater facilities (Ziylan & Ince, 2011). The NSAIDs diclofenac, naproxen and ibuprofen have been detected in surface waters (Table S1) (Kay et al., 2017; Osorio et al., 2016), seawaters (Gros et al., 2012) (Table S3), groundwaters (López-Serna et al., 2013) (Table S2), drinking water (Gaffney et al., 2015; Padhye et al., 2014) (Table S4), wastewater and sludge (Kay et al., 2017; Kumirska et al., 2015; Petrie et al., 2016) (Table S6 and S7) and sediments (Biel-Maeso et al., 2017; Osorio et al., 2016) (Table S5).

Several studies reported the effect of NSAIDs in non-target organisms. In a study conducted by Xia and collaborators (2017), a significant hatch delay of zebra fish (*Danio rerio*), due to the suppression of overall embryo motion, was observed after an exposure to ibuprofen (500 μg L^{-1}) and diclofenac (5 μg L^{-1} and 500 μg L^{-1}) (Xia et al., 2017). Furthermore, Kwak and collaborators (2018), described a reduction in the reproduction of the crustaceans *Daphnia magna* and *Moina macrocopa* due to chronic exposure to naproxen (Kwak et al., 2018). In addition, the same authors reported a decrease in the survival of juvenile *Oryzias latipes* fish exposed to 5 mg L^{-1} of naproxen (Kwak et al., 2018). Moreover, ibuprofen was found to cause nephrotoxicity in the south American catfish, *Rhamdia quelen* (Mathias et al., 2018). When exposed to four different NSAIDs (ibuprofen (racemic and S-(+)- ibuprofen), ketoprofen and aspirin), the green algae *Scenedesmus obliquus* showed growth inhibition, severe damage on cellular structures, significant effects on photosynthesis and on the PSI-PSII photosynthetic electron transport chain as well as on carbon assimilation and photorespiration (Wang et al., 2020). Authors also reported that ketoprofen was the NSAID that exerted higher toxicity on *Scenedesmus obliquus,* suggesting that it could be related to its high liposolubility and bioavailability (Wang et al., 2020).

1.4.3 Antidepressants

Antidepressants are an important group of pharmaceuticals designed to treat psychological disorders and extensively used throughout the world. Antidepressants can be divided into four major classes: monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), norepinephrine reuptake inhibitors (SNRIs) and selective serotonin reuptake inhibitors (SSRIs) (Lajeunesse et al., 2008; Sehonova et al., 2019). From these, SSRIs are the most prescribed class of antidepressants (Lajeunesse et al., 2008; Shaliutina-Kolešová et al., 2020). SSRIs have been used since the 80s (Schultz &

Furlong, 2008) and continue to be the first choice in treating depression due to their therapeutic effectiveness and higher acceptability and safety comparing with other groups of antidepressants (e.g. TCAs or SNRIs) (Gołyszny & Obuchowicz, 2019). Antidepressants are used to treat clinical depression, obsessive - compulsive disorder, panic disorder, attention-deficit disorder, eating disorder (nervous bulimia and compulsive ingestion) and social phobia (Bulik et al., 2012; Schultz & Furlong, 2008). The SSRIs fluoxetine, paroxetine, citalopram and sertraline, and the SNRIs venlafaxine and duloxetine are examples of the most prescribed antidepressants in the current days (Fong & Ford, 2014; Shaliutina-Kolešová et al., 2020; Silva et al., 2015a). SSRIs, SNRIs and TCAs act through modulation of serotonergic, dopaminergic or noradrenergic neurotransmission (Fong & Ford, 2014).

Consumption of antidepressants has been increasing in the last years. In fact, the reports from OECD show that between 2000 and 2017 the consumption of antidepressants doubled in OECD countries (OECD, 2019). Moreover, data from 2017 showed that Iceland and Canada presented the highest consumption of antidepressants, while Latavia, Korea, Hungary and Estonia presented the lowest consumption values (OECD, 2019). In Portugal, around 300 000 packages of antidepressants were prescribed in 2001, and in 2016, almost 8 000 000 packages of antidepressants were consumed (data does not include the antidepressants prescribed in hospital facilities) (INFARMED). According to the 2019 OECD report, Portugal was the 4th country with the highest level of antidepressants consumption between 2000 and 2017, being only surpassed by Iceland, Australia, Canada and UK (OECD, 2019), a tendency also reported in the 2015 OECD report (OECD, 2015). In the last years, the continuous growth of antidepressants consumption was linked to economic crises. Specifically, in Portugal, antidepressant consumption went up by 30% between 2007 and 2012, but this level was lower than the 60% growth rate observed between 2002 and 2007 years (OECD, 2014). In Spain, the consumption of antidepressants per capita increased by 23% between 2007 and 2012, even though this increase was lower than the 44% growth rate observed between 2002 and 2007 (OECD, 2014). During this period, both countries faced financial and economic adversities, expressed by an increase in unemployment (3% for Portugal and 12% for Spain), the fear of losing the jobs and, significant reduction or freezing of salaries, among others (Karanikolos et al., 2013). According to Karanikolos et al. (2013), the economic crisis had a significant impact on mental health, translating in an increase of antidepressants consumption (Karanikolos et al., 2013). Nevertheless, in Germany, one of the countries less affected by economic crises, a quick rise in antidepressants consumption (over 12%) was observed between 2007 and

2012 (OECD, 2014). Data on antidepressants consumption may be, however, underestimated (based on prescribing trends), as some antidepressants, like fluoxetine, are off patent being more difficult to track (Schultz et al., 2010). Nowadays, with the coronavirus SARS-CoV-2 (COVID-19) pandemic situation, a new increase in the consumption of these pharmaceutical products can be expected. COVID-19 pandemic is a challenge for both physical and mental health for human race, as well as to economy and social life (Wasserman et al., 2020). Implementation of lockdown measures, including work disruptions, school closures and physical distancing, might increase social isolation and loneliness feeling, both in turn associated with an increased anxiety, depression and suicidal behavior (Wasserman et al., 2020).

Antidepressants can enter in the aquatic ecosystems through inefficient treatment of wastewaters from municipalities, hospitals, and pharmaceutical industries and through improper disposal. The presence of antidepressants in different environments (wastewaters, surface waters and/or drinking water) was reported in several studies (Benotti & Brownawell, 2009; Gros et al., 2012; Petrie et al., 2016). Antidepressants such as fluoxetine, paroxetine, sertraline, venlafaxine and citalopram have been detected in surface waters (Gros et al., 2012; Osorio et al., 2016) (Table S1), wastewaters (Gros et al., 2012; Petrie et al., 2016; Petrović et al., 2014) (Table S6), sludge (Petrie et al., 2016) (Table S6) and sediments (Osorio et al., 2016) (Table S5). Some of them were also detected in seawater (Gros et al., 2012) (Table S3) and groundwater (López-Serna et al., 2013) (Table S2). For instance, venlafaxine was detected in surface waters at concentrations ranging between 1.15 and 575 ng L^{-1} (Gracia-Lor et al., 2011; Osorio et al., 2016) and at lower concentrations in seawater (52 ng L^{-1}) (Gros et al., 2012), sediments (0.05 – 1.94 ng g^{-1}) (Osorio et al., 2016) and sludge (37.9 ng g^{-1}) (Petrie et al., 2016). The antidepressant paroxetine was reported in surface waters at concentrations ranging between 0.27 and 40 ng L^{-1} (Gros et al., 2012; Osorio et al., 2016) and groundwater at similar concentrations (5.17 – 30.2 ng L^{-1}) (López-Serna et al., 2013). Like for other pharmaceuticals, a range of concentrations of antidepressants can be found in the same environment (Tables S1-S7). This variation can be related with the consumption profile associated with the site where the samples are being collected; detection methods used for their detection and, more important, with the behavior of each antidepressant in the environment.

Antidepressants can induce effects in living organisms even at very low concentrations, so their presence in the environment is of high concern (Schultz et al., 2010; Simpson et al., 2007). This problem can be exacerbated through the chronic administration of antidepressants, which may lead to a higher and continuous environmental input and

exposure to these compounds (Silva et al., 2012). For example, serotonin is known to regulate several physiological processes in fish, mollusks and protozoans (Silva et al., 2012, and references therein). Several studies have addressed the adverse effects of antidepressants in organisms. Johnson and collaborators (2007), showed that the SSRIs fluoxetine, fluvoxamine and sertraline presented toxic effects on algae, with IC10 values ranging from 4.6 to 6100 μg L^{-1} (depending on the algae species) after 96 h acute growth inhibition (Johnson et al., 2007). Sehonova and collaborators (2019), studied the effects of three antidepressants, venlafaxine, amitriptyline and sertraline, on early life stages of non-target aquatic organisms (*Danio rerio* and *Xenopus tropicalis*), showing swimming alterations at high antidepressants concentration (i.e., concentrations higher than those found in the environment) (Sehonova et al., 2019). In addition, lethal and sublethal effects were observed in the embryos of both species for the highest tested amitriptyline concentration. The study also reported that, at an environmental relevant concentration, the three antidepressants were suspected to have an effect on mRNA (messenger Ribonucleic Acid) expression of genes related to heart, eye, brain and bone development (Sehonova et al., 2019). Nowakowska and collaborators (2020), showed that exposure of zebra fish larvae to selected antidepressants (paroxetine, sertraline, fluoxetine and mianserin) caused an increasing rate of abnormal embryo and larvae development, accelerating the hatching time and influencing the total hatching rate (Nowakowska et al., 2020). The authors also reported a decrease of proliferation of hepatocytes in larvae previously subjected to paroxetine, mianserin, sertraline (10 μg L^{-1}) and also to a mixture of all antidepressants at 25 μg L^{-1} (Nowakowska et al., 2020).

1.4.4 Blood lipid lowering agents

Blood lipid lowering agents are commonly prescribed to treat diseases related to cardiovascular disorders (Neuvonen et al., 2006). There are two main groups with different functions: statins and fibrates. Statins are used mainly to suppress cholesterol biosynthesis by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Pahan, 2006). Several statins are currently available in the market, such as atorvastatin, simvastatin, lovastatin, pitavastatin, fluvastatin, cerivastatin, rosuvastatin and pravastatin (Miao et al., 2018; Nováková et al., 2008). On the contrary, fibrates are used to reduce plasma levels of fatty acids and triacylglycerol by stimulating β-oxidation of fatty acids, mostly in peroxisomes and partly in mitochondria (Pahan, 2006). Examples of some well-known fibrate drugs are fenofibrate, bezafibrate, ciprofibrate and gemfibrozil (Pahan, 2006).

Lipid lowering agents are widely prescribed worldwide and their consumption has been growing. Prescription of cholesterol-lowering drugs has raised between 2000 and 2013, in which Slovak Republic, UK and Australia were the countries with the highest consumption per capita in 2013 (OECD, 2015). The same tendency was reported in the latest OECD report, in which UK, Denmark and Belgium had the highest consumption per capita in 2017 (OECD, 2019). In the case of Portugal, more than 10 million packages were consumed in 2016, being the statins simvastatin, atorvastatin and rosuvastatin the most consumed ones (INFARMED). Fenofibrate and bezafibrate were also widely consumed in Portugal in the same year but in smaller proportions compared to statins (INFARMED).

Like other pharmaceuticals, lipid lowering agents have been detected in different environmental compartments. As an example, bezafibrate was detected in effluent samples of three different wastewater facilities in Spain, at concentrations ranging between 2 and 132 ng L^{-1} (Table S7), and in three estuarine environments (water samples) at concentrations ranging between 2 and 67 ng L^{-1} (Mijangos et al., 2018) (Table S1). Surface waters in Portugal were found to be contaminated with low levels of bezafibrate and gemfibrozil (11.86 - 15.52 ng L^{-1} and 6.69 – 10.34 ng L^{-1}, respectively) (Pereira et al., 2017). In Mexico, bezafibrate and gemfibrozil were detected in surface waters at levels ranging between 265 - 2100 ng L^{-1} and 9 - 380 ng L^{-1}, respectively (Rivera-Jaimes et al., 2018). Fibrates have also been detected in groundwaters (López-Serna et al., 2013) (Table S4) and seawaters (Gros et al., 2012) (Table S2). Regarding the environmental presence of statins, few studies have been dedicated to this issue despite the increase of their consumption. In a study conducted by Langford and Thomas (2011), simvastatin was not detected in surface waters nor in sediments (concentrations below the detection limit) nevertheless, its metabolite, simvastatin hydroxy carboxylic acid, was present in surface waters (27 - 66 ng L^{-1}) and in sediments (2 - 4 ng g^{-1}) (Langford & Thomas, 2011). In the same study, the metabolites of atorvastatin, *p*-hydroxy atorvastatin and *o*-hydroxy atorvastatin, were detected in wastewaters at levels ranging between 83 - 233 ng L^{-1} and 60 - 169 ng L^{-1}, respectively, whereas the parent compound was detected at lower levels (23 - 37 ng L^{-1}). Atorvastatin and rosuvastatin were detected in 11 WWTPs in Ontario, in both influent and effluent samples (Lee et al., 2009). Atorvastatin was detected at concentrations ranging between 72 - 263 ng L^{-1} and 10 - 122 ng L^{-1} in influent and effluent samples, respectively, and rosuvastatin was detected at 34 - 604 ng L^{-1} and 190 - 552 ng L^{-1} in influent and effluent samples, respectively (Table S7).

The effects of blood lipid lowering agents in the environment and living organisms have been reported in some studies. Mijangos et al. (2018), evaluated the environmental

risk of different pharmaceuticals in wastewater effluents and estuarine samples, by analysing the chronic and acute toxicity of the selected pharmaceuticals (Mijangos et al., 2018). In terms of chronic toxicity, authors showed that: a) bezafibrate, alongside with diclofenac and sulfadiazine, and b) bezafibrate and diclofenac displayed the most negative impact in the wastewater effluents and estuarine sediments, respectively (risk quotient (RQ) > 1) (Mijangos et al., 2018). However, in terms of acute toxicity, bezafibrate presented a RQ < 1 for both matrices (Mijangos et al., 2018). In a study with mussels (*Mytilus edulis*), atorvastatin induced an increasing of basal metabolic rate and a reduction of energy reserves (Falfushynska et al., 2019). In addition, the authors also reported that atorvastatin can act as a metabolic disruptor and chemosensitizer in *M. edulis* (Falfushynska et al., 2019). Barros and collaborators (2018) showed that simvastatin exposure led to a reduction in cholesterol/triglyceride levels and altered key genes expression in zebra fish (Barros et al., 2018).

1.5 Analytical methods for detection of pharmaceuticals in the environment

The development of new analytical methods and improvement of the old ones, combined with new advanced techniques, allowed low detection levels of pharmaceuticals in environmental matrices. Gas chromatography - tandem mass spectrometry (GC-MS/MS) and liquid chromatography - tandem mass spectrometry (LC-MS/MS) are the top technologies used for the detection and monitoring of CECs in the environment, allowing the detection of CECs at ng L^{-1} and identification of both parent compounds and associated metabolites. The selection of the analytical method most suitable for the chemical target compound must consider its physical and chemical properties. For instance, compounds that are heat-unstable and non-volatile should be detected by LC-MS/MS, whereas for volatile compounds GC-MS/MS is the most appropriate instrument. Some compounds can be analysed by both methods, but the detection sensitivity differs.

LC-MS/MS has been used to detect several classes of pollutants in environmental matrices that present medium-high polarity and very low to none volatility (Hernández et al., 2014; Togunde et al., 2012). The use of this technique has increased due to its high sensitivity and selectivity for the measurement of organic pollutants in the environment at ng L^{-1} levels (Petrovic et al., 2010).

LC-MS/MS allows simultaneous determination of different types of compounds in high polluted aquatic environments (Madureira et al., 2010), in a unique run, within a shorter time period and with lower costs (Hernández et al., 2014). However, the detection of

transformation products or active metabolites resultant from the target pollutants is also of great relevance as these can have a higher negative effect in the environment than the parent compound (Llorca et al., 2016). Nevertheless, the detection of these metabolites, and sometimes of the pollutant itself, can be very difficult due to the unavailability of chemical standards for all pollutants (Llorca et al., 2016). In this regard, several technologies can be coupled to identify the target compounds, specifically hybrid triple quadrupole-linear ion trap mass spectrometer (QqLIT-MS/MS) (Petrović et al., 2014) and high-resolution mass spectrometry (HRMS) (Gros et al., 2014). In addition, liquid chromatography techniques have been improved in the past few years and, in these days, ultra-high-performance liquid chromatography (UHPLC) has been widely used. UHPLC provides a faster analysis allied to a superior resolution compared with traditional high-performance liquid chromatography (HPLC) techniques (Guillarme et al., 2010).The main difference between HPLC and UHPLC is the column material particle size used in the latter (less than 2-µm) (Taleuzzaman et al., 2015). UHPLC has lower costs, shorter run time, higher selectivity and sensitivity and reduced consumption of solvents (Taleuzzaman et al., 2015).

Generally, analytical techniques are combined with modern extraction and clean up procedures (Guerra et al., 2014 and references therein) to assure a better analysis in terms of sensitivity and removal of matrix interferences. Solid phase extraction (SPE), liquid-liquid extraction (LLE), solid phase microextraction (SPME) and liquid–liquid membrane extraction (LLME) are the most used extraction methodologies, where pre-concentration of the sample and extract clean-up is obtained simultaneously from complex aquatic matrices (Pavlović et al., 2007; Wang & Wang, 2016). Usually, these methods require optimization of the measurement parameters and can be time consuming, however they are essential to obtain a good performance (Wang & Wang, 2016).

1.6 Removal of pharmaceuticals in wastewater treatment plants and factors that can affect their removal

Wastewater treatment plants (WWTPs) were designed to efficiently remove suspended solids, organic matter, nutrients and pathogens (Guerra et al., 2014). However, their efficiency to remove micropollutants, such as pharmaceuticals, is generally very low as they were not designed to remove this type of compounds (Caracciolo et al., 2015; Guerra et al., 2014). Pharmaceuticals can go through conventional wastewater treatments unaltered because of their moderate to high solubility and their resilience to degradation during biological and chemical processes (Blair et al., 2013; Feng et al., 2013).

The inefficient removal of pharmaceuticals in WWTPs can be related to several factors inherent to treatments and operational conditions. For instance, one major factor that cannot be controlled is the weather conditions. The removal efficiencies can be lower during winter due to heavy rainfalls and lower water temperature, which can lead to a decrease in biodegradation kinetics. Moreover, pollutant concentration can affect the removal rates. For instance, removal rates of some anti-inflammatories, antibiotics and antidepressants can decrease in winter, since usually in this time of the year the consumption of these compounds increases due to weather-associated health problems, such as flue or rheumatic pains (Tixier et al., 2003; Yu et al., 2013), and seasonal human depression (depression or disorder that have more incidence during specific times of the year, usually in autumn and winter) (Kurlansik & Ibay, 2012; Lurie et al., 2006). Vieno and collaborators (2005), reported that the total concentration of the pharmaceuticals ibuprofen, naproxen, ketoprofen, diclofenac and bezafibrate in the effluents of a sewage treatment plant was 3-5 times higher in winter (about 2500 ng L^{-1}) than in the other seasons (about 500−900 ng L^{-1}) (Vieno et al., 2005). On the other hand, Guerra et al. (2014) studied six WWTPs and found seasonal differences in terms of pharmaceuticals concentrations in the effluents for only one WWTP (Guerra et al., 2014).

Other factors such as pH, hydraulic retention time (HRT), sludge retention time (SRT), food to microorganisms (F/M) ratio and configuration of the WWTPs, can have a key role in the removal efficiency of pharmaceuticals (Krzeminski et al., 2019; Verlicchi et al., 2012; Ziylan & Ince, 2011). HRT and SRT control the reaction time and loading, affecting biomass activity and the adaptation of different microbial communities (McAdam et al., 2010; Verlicchi et al., 2012). It is expected that higher HRT and SRT lead to higher biodegradation of contaminants, because higher retention times can promote the development of slowly growing bacteria and, thus, stimulate more microbial diversity with wider physiological capabilities (Clara et al., 2005). Indeed, Clara et al. (2005) showed that the removal of different emerging pollutants, including the pharmaceuticals bezafibrate and ibuprofen, was enhanced at higher SRT but, for other compounds such as carbamazepine, the same effect was not observed, as carbamazepine was not degraded during the treatment (Clara et al., 2005). Guerra and collaborators (2014), reported that the efficient removal of several pharmaceuticals and personal care products (PPCPs) was strongly related with summer temperatures, HRT longer than 16 h and nitrifying activity (Guerra et al., 2014). Moreover, Fernandez-Fontaina et al. (2012), studied the influence of HRT, SRT, temperature and nitrifying activity on the biodegradation of several contaminants, including different classes of pharmaceuticals, in a pure nitrifying reactor (Fernandez-Fontaina et al.,

2012). The authors observed that the biodegradation rates of ibuprofen, naproxen, trimethoprim, roxithromycin, erythromycin and fluoxetine (and other emerging pollutants) increased with an increase in nitrogen loading rates, being the ammonium monooxygenase enzyme (AMO) responsible for co-metabolic biodegradation (Fernandez-Fontaina et al., 2012). They also reported that contaminants with slow or intermediate degradation kinetics, like fluoxetine or antibiotics, are expected to have lower biodegradation efficiencies when HRTs are lower and/or loading rates are higher (Fernandez-Fontaina et al., 2012). Despite these results, other authors reported that there was no clear relationship between removal efficiencies and SRT/HRT for the antibiotics ciprofloxacin, ofloxacin and norfloxacin, β-blockers (Vieno et al., 2007) and carbamazepine (Joss et al., 2005; Vieno et al., 2007).

The pH is another parameter that can highly influence the removal of pharmaceuticals, as under different pH conditions these compounds can change their ionic form, becoming neutral, cationic, anionic or zwitterionic. Thus, the physical-chemical and biological properties of pharmaceuticals, such as toxicity, activity, sorption and photo-sensitivity, will vary with the pH of the medium (Verlicchi et al., 2012 and references therein). Antibiotics are one of the groups that can be strongly affected by pH variations, especially the antibiotics ciprofloxacin, tetracycline and penicillin G (Verlicchi et al., 2012). A study, on the removal of several pharmaceuticals in a submerged membrane bioreactor (MBR) at different pH values (between 5 and 9), was conducted and showed a strong influence of pH in the removal of ibuprofen, ketoprofen, diclofenac and sulfamethoxazole, with the highest removals being achieved at pH 5 (Tadkaew et al., 2010). On the other hand, for the same pH range, no significant influence of pH on the removal of the anti-lipidic bezafibrate, in an activated sludge system was observed (Sui et al., 2016). Baena-Nogueras and collaborators (2017) showed that pH has a key role in the photodegradation of many pharmaceutical compounds (Baena-Nogueras et al., 2017). In fact, the authors observed that photodegradation of the analgesic acetaminophen was higher at pH 4 or 9 but for other pharmaceuticals like diclofenac, ketoprofen and ibuprofen (NSAIDs pharmaceuticals), no significant changes were observed (Baena-Nogueras et al., 2017).

The F/M ratio can affect organic removal efficiency, microbial composition and sludge properties (Liu et al., 2012; Wu et al., 2018). Lower F/M ratios combined with higher retention times can lead to an increase in biodiversity and enhance the degradation of pollutants by co-metabolism (Gobel et al., 2007; Sipma et al., 2010). In addition, low substrate availability can induce microorganisms to use poor degradable compounds as carbon sources (Verlicchi et al., 2012).

Treatment configuration also has a huge impact in the removal efficiencies of pharmaceuticals in WWTPs (Krzeminski et al., 2019). Different biochemical environments (aerobic, anaerobic or anoxic conditions) can promote or inhibit the removal of certain pharmaceuticals. The microbial communities present in each type of environment are completely different and have different metabolic mechanisms, which can influence the biodegradation of the pollutant. Alvarino and co-authors (2014) studied the fate of 16 PPCPs in an aerobic conventional activated sludge reactor (CAS unit) and in an upflow anaerobic sludge blanket reactor (UASB reactor), testing different operational periods (Alvarino et al., 2014). The authors showed that under aerobic conditions (CAS unit), higher removal efficiencies were obtained for most of the 16 PPCPs (Alvarino et al., 2014), except for sulfamethoxazole and trimethoprim. However, in the CAS unit, carbamazepine, diclofenac, diazepam and trimethoprim had removal efficiencies below 10% in all tested periods (Alvarino et al., 2014). In addition, authors also reported that ,under anaerobic conditions (UASB reactor), sulfamethoxazole and trimethoprim presented higher removal efficiency, while removal efficiency of naproxen was similar in both reactors (Alvarino et al., 2014). Suarez and collaborators (2010), reported improvements in ibuprofen and diclofenac removal from wastewater effluents only when specific types of bacteria were able to grow (Suarez et al., 2010).

The fate of pharmaceuticals during wastewater treatment processes can also differ with the therapeutic class. In a study, where the fate of several pharmaceuticals in WWTPs was evaluated, it was reported that anti-inflammatories and analgesics were susceptible to biodegradation in a conventional biological treatment, whereas they were not biodegraded during a chemically assisted primary treatment (Guerra et al., 2014). The study also showed that antibiotics and antifungal compounds were highly resistant to both treatments being detected at high concentrations in the treated effluent and sludge (Guerra et al., 2014).

Despite all these factors, the removal efficiencies of pharmaceuticals in WWTPs can change in different facilities with the same treatment or even in the same facility and in the same day. These changes can occur due to physicochemical properties of pharmaceuticals, effluent composition (microbial community, wastewater composition and other elements that can be present and improve or inhibit degradation) and different WWTPs configurations like biological treatment configuration and operational parameters. For instance, the same pollutant can have different removal rates in the same type of biological treatment and even in the same facility. A study conducted by Roberts et al. (2016) showed that for some target pharmaceuticals removal efficiency varied between sampling campaigns at relatively constant sewage treatment conditions (Roberts et al., 2016).

All the mentioned factors can be optimized, and more attention is needed regarding this topic. An improvement in these parameters can lead to better removal efficiencies of some pharmaceuticals and of other organic compounds in wastewater treatment facilities without changing the type of treatment, leading to lower emissions of pharmaceuticals in the environment.

Several technologies have emerged in the last years to tackle pharmaceuticals contamination and improve their removal in WWTPs, since these are the main input of pharmaceuticals into the environment. By solving the problem in wastewater facilities, a large amount of pharmaceuticals can be removed before entering the environment. Chemical based technologies have been developed, in which advanced oxidation processes are the most studied (Wang & Wang, 2016). Chlorination, ozonation, UV treatment, electrochemical oxidation and Fenton and photo Fenton oxidation are examples of advanced oxidation processes (Kanakaraju et al., 2018; Wang & Wang, 2016). Physical based technologies have also been explored, with adsorption processes (activated carbon, carbon nanotubes) being the most commonly known (Wang & Wang, 2016).

1.7 Bioremediation processes as a new remediation technology

The degradation of pollutants by microbial communities is one of the most important mechanisms for removing these compounds from the environment. Microbial communities are essential degraders of organic matter and, at the same time, they provide nutrients to other organisms in the food chain (Caracciolo et al., 2015). Therefore, they are extremely important for the function, maintenance, quality state and natural depuration of ecosystems. When a xenobiotic enters the environment, changes in local microbial communities can occur and, consequently, ecosystems processes can also change (Lahti & Oikari, 2011). Microbial communities can degrade organic contaminants by metabolic and co-metabolic reactions, with the latter being the most important for the elimination of pollutants (Caracciolo et al., 2015; Lahti & Oikari, 2011). Bioremediation technology relies on the metabolic capacity of microorganisms to degrade pollutants, taking advantage of the natural detoxification processes (Megharaj et al., 2011). For that, it is crucial the selection and isolation of natural degrading microorganisms to develop microbial inocula able to degrade target contaminants, with minimal long-term influence in the contaminated sites (Paul et al., 2005). This technology has gained attention due to the high abundance and diversity of microorganisms in nature, their highly diverse catalytic mechanisms and capacity to function in and adapt to a multitude of conditions (Megharaj et al., 2011).

A consortium of microorganisms is usually more effective for the degradation of contaminants than single bacterial strains, as cooperative interactions or synergistic effects among different bacteria can play a crucial role in the degradation of these compounds (Wang & Wang, 2016; Zhao et al., 2015). Nevertheless, bioremediation of a contaminated site is only possible if the toxicity of the contaminant does not inhibit microbial activity (Caracciolo et al., 2015).

Bioremediation has been reported as an efficient method for the remediation of organic pollutants such as hydrocarbons (Ghazali et al., 2004; Tahhan et al., 2011; Wang et al., 2019), pesticides (Diaz et al., 2016; Kumar & Philip, 2006), polychlorinated biphenyls (Egorova et al., 2013) and pharmaceuticals (Alexandrino et al., 2017; Fernandes et al., 2020). This technology is based on three strategies: biostimulation, bioaugmentation or a combination of both. Biostimulation aims to stimulate local and/or introduced microbial community by adding an inorganic nutrient cocktail (mainly containing nitrogen and phosphorus) to the contaminated site, in order to avoid metabolic restrictions (Abed et al., 2014; Lee & Merlin, 1999; Yu et al., 2005). Bioaugmentation comprises microbial inoculation of degrading microorganisms to the contaminated site in order to enhance the biodegradation of the target pollutant(s) (Abed et al., 2014; Lee & Merlin, 1999; Yu et al., 2005). Combination of both processes, bioaugmentation and biostimulation, has been explored since the addition of a nutrients cocktail for stimulation of the natural and bioaugmented community is a crucial feature for the success of the bioremediation process.

1.7.1 Biodegradation of pharmaceuticals by single strains

Biodegradation of pharmaceuticals using single bacterial strains isolated from different environments has been investigated. Ofloxacin, norfloxacin and ciprofloxacin was reported to be biodegraded, individually or as mixture, in the presence of sodium acetate as an additional carbon source (Amorim et al., 2014), by a bacterial strain identified as *Labrys portucalensis* F11 (Carvalho et al., 2005), isolated from an industrially contaminated site in Northern Portugal. Complete degradation by *L. portucalensis* F11 of 2 µM racemic fluoxetine in 30 days was reported by Moreira et al. (2014) however, when the racemic compound was supplied at 4 µM, the (R)-enantiomer was preferentially degraded over the (S)-enantiomer, with 80% of (S) - fluoxetine and 97% of (R) - fluoxetine being degraded (Moreira et al., 2014). These findings indicated that enantiomeric pharmaceuticals are not biodegraded at the same extent. The authors also reported a decrease in the removal rate of fluoxetine with the increase in its concentration, a result also reported by Amorim and collaborators (Amorim et al., 2014). Complete dehalogenation of moxifloxacin (up to 7.5

µM) by *L. portucalensis* F11 strain, with sodium acetate as co-substrate, was reported by Carvalho and collaborators (Carvalho et al., 2016). This microorganism also showed to be capable of fully removing 34 µM of diclofenac from the culture medium in co-metabolism with sodium acetate (5.9 mM) in 25 days (Moreira et al., 2018). In addition, no chlorinated compound was found at the end of the experiment, indicating complete dehalogenation of diclofenac (Moreira et al., 2018). The degradation of diclofenac and carbamazepine (at 10 mg L^{-1}) by bacterial strains isolated from an activated sludge from a municipal WWTP was also tested (Bessa et al., 2017). The authors reported a *Brevibacterium* sp. D4 strain capable of removing 90% of diclofenac and *Starkeya* sp. C11 and *Rhizobium* sp. C12 strains capable of removing 32% of carbamazepine, both in the presence of acetate as a supplementary carbon source (Bessa et al., 2017).

The biodegradation of sulfamethazine with the bacterial strains *Geobacillus* sp. strain S-07 and the type strain *Geobacillus thermoleovorans* was also investigated (Pan et al., 2017). In 24h of experiment, strain S-07 revealed to be capable of removing more than 95% of the antibiotic in co-metabolism with glucose, while the type strain *G. thermoleovorans* only removed 30% of the compound (a percentage that also includes abiotic degradation) (Pan et al., 2017). In another study, the authors investigated the degradation of ciprofloxacin by the thermophilic bacterium *Thermus* sp. strain C419 isolated from the sludge of an antibiotics producing factory (Pan et al., 2018). The authors tested different temperatures ranging from 65ºC to 80ºC and found that ciprofloxacin was better degraded at 70ºC (Pan et al., 2018). In addition, the authors performed biodegradation experiments in co-metabolism with sodium acetate and observed that acetate promoted bacterial growth and enhanced the degradation of ciprofloxacin, being removed around 60% of the antibiotic after 5 days of exposure (Pan et al., 2018). It was also assessed the potential of three bacterial isolates, *Ochrobactrum* sp. SA1, *Labrys* sp. SC11 and *Gordonia* sp. SCD14, to degrade sulfamethoxazole (Mulla et al., 2018). The three isolates were obtained from a culture enriched from wastewater and sludge inoculum and with 6 mg L^{-1} of the target compound as sole carbon source and were able to partially degrade 5 mg L^{-1} of sulfamethoxazole (45.2%, 62.2% and 51.4%, respectively) (Mulla et al., 2018). Zhang and collaborators (2013) tested the ability of three strains, one *Stenotrophomonas* sp. and two *Pseudomonas* sp., isolated from a paracetamol-degrading microbial aggregate growing in a lab-scale airlift sequencing batch reactor, to degrade paracetamol (Zhang et al., 2013). The strains tested were used as a consortium or as single strains. The three strains were able to individually degrade the pharmaceutical, however, high concentrations of the pharmaceutical were found to be toxic and to inhibit the degradation of paracetamol, i.e.,

degradation by *Stenotrophomonas* sp. was inhibited at 600 mg L^{-1} whereas degradation by the strain *Pseudomonas* sp. f2 was inhibited in the presence of 3.000 mg L^{-1} of paracetamol (Zhang et al., 2013). However, *Pseudomonas* sp. fg-2 was able to degrade up to 2,000 mg L^{-1} in 45h (Zhang et al., 2013). In addition, they also showed that the consortium formed by the three strains was more efficient than the strains individually, since this mix was able to completely degrade paracetamol at concentrations up to 4.000 mg L^{-1}, indicating possible synergistic interactions between the three isolates in the degradation of the pharmaceutical (Zhang et al., 2013).

1.7.2 Biodegradation by bacterial consortia

Studies exploring the potential of microbial consortia to degrade pharmaceuticals have also been performed. Hay (2001) enriched a consortium from activated sludge able to use triclosan as sole carbon source (Hay, 2001). However, the strains recovered from the enriched consortium (composed by the genera *Pseudomonas*, *Alcaligenes*, *Rhodanobacter*, *Agrobacterium* and *Sphingomonas*, all belonging to the Proteobacteria phylum) were not able to use triclosan as a sole carbon source in liquid medium, either individually or combined. In another study, the role of different types of bacteria (ammonia oxidizing and heterotrophic bacteria) in the degradation of trimethoprim and 17α-ethinylestradiol was evaluated (Khunjar et al., 2011). A mixed culture, of both ammonia oxidizing and heterotrophic bacteria (composition not disclosed), was proved to enhance the removal of 17α-ethinylestradiol (Khunjar et al., 2011). Furthermore, Alexandrino and collaborators (2017) investigated the biodegradation of three enrofloxacin and ceftiofur concentrations (1, 2 and 3 mg L^{-1}), either alone or in mixture, using microbial consortia obtained from rhizosediment of plants from constructed wetlands (Alexandrino et al., 2017). In that study, the authors reported complete removal of ceftiofur in all experiments, even in the presence of enrofloxacin. However, enrofloxacin never reached complete removal (around 40 - 55%), with the increasing antibiotic concentration being a limiting factor, as also reported by other authors (Amorim et al., 2014; Moreira et al., 2014). The authors additionally found that the predominant microorganisms resulting from acclimation with the target antibiotics belonged to the phyla Proteobacteria (e.g., *Achromobacter*, *Variovorax* and *Stenotrophomonas* genera) and Bacteroidetes (e.g., *Dysgonomonas*, *Flavobacterium* and *Chryseobacterium* genera) (Alexandrino et al., 2017). Facey et al. (2018) showed that diclofenac was removed in 7 days by two microbial consortia native from forest soils in Germany (microbial composition not identified), when present at concentrations up to 0.1 g L^{-1} (Facey et al., 2018). Moreover, it was reported the biodegradation of naproxen by

microbial communities (composition not revealed) of three types of agricultural soil (sandy loam, loam and silt loam) and showed that this compound was rapidly biodegraded and mineralized (Topp et al., 2008). More recently, the capability of five bacterial consortia enriched from sludge or estuarine sediment to degrade 1 mg L^{-1} of paroxetine and bezafibrate, under different incubation conditions (static and agitation), was described (Duarte et al., 2019; Fernandes et al., 2020). In this study, bacteria affiliated with the phylum Proteobacteria were dominant in all consortia, with the genus *Pseudomonas* being the most abundant (Fernandes et al., 2020). Nonetheless, bacteria belonging to the genera *Acinetobacter*, *Dyadobacter* (Bacteroidetes) and *Microbacterium* (Actinobacteria), among others, were also found (Fernandes et al., 2020).

All these studies clearly show that the use of bacterial strains or bacterial consortia can be an option to remove/degrade pharmaceutical compounds from the environment. Despite the important information that these studies provide, it is very difficult to describe a common pattern in terms of degradation of these compounds, since a wide variation in removal efficiencies across/between therapeutic classes, treatment processes and even between different studies using the same pharmaceutical compound were observed.

1.7.3 Factors affecting biodegradation process

In a biological treatment, several processes as volatilization, adsorption, and biodegradation can occur (Wang & Wang, 2016). Biodegradation/biotransformation together with adsorption are the processes that have the highest role in the degradation of pharmaceuticals during biological treatment in wastewater treatment facilities (Santos et al., 2013). The physicochemical properties of the pharmaceutical compounds determine whether they will be degraded or adsorbed to the sludge. Biodegradation of these compounds is also dependent on the abundance of microbial degraders in the treatment system and can be very low in systems poor in microbial degraders (Wang & Wang, 2016). In addition, pharmaceuticals degradation can be affected by interactions with other compounds (antagonistic effect) (Vasquez et al., 2014) or interactions among microorganisms (synergistic effect) (Zhao et al., 2015). These synergistic or antagonistic effects can improve or inhibit the degradation of pharmaceuticals, being a potential explanation for the different removal efficiencies obtained with the same treatment.

To evaluate and compare the biodegradability of pharmaceuticals it is necessary to take into account the intrinsic differences in the chemical structure of each compound, like the presence of sugar moieties or of halogens on the compound structure, which can render the compound more or less biodegradable (Kümmerer & Al-Ahmad, 1997). Thus,

pharmaceuticals within the same therapeutic class but with different chemical structures can have different biodegradation rates, since biodegradation processes engage enzymatic reactions that are chemically specific (Onesios et al., 2009). In addition to the factors presented here, there are others that can also affect the biodegradation of pharmaceuticals and explain the discrepancies in the removal rates observed for the same compound. The first, and a very important one, is the pharmaceutical concentration. Different concentrations lead to different removal efficiencies that cannot be compared. Moreover, too high pharmaceutical concentrations can inhibit the microbial community and exert a toxic effect on microorganisms (Onesios et al., 2009). Another factor, which is related with the first one, is the concentration of the primary substrate. Pharmaceuticals can potentially be used as a primary substrate, *i.e.*, they can be utilized by microbial communities as a carbon and energy source, depending on the concentration of the pharmaceutical. If the concentration is very low, it may not be sufficient to induce specific degrading enzymes leading to the compound biodegradation preferentially through co-metabolism (Ternes & Joss, 2006). So, the fact that pharmaceuticals can be used as a primary substrate or as a co-substrate can contribute to differences in biodegradation rates. The third factor that can affect biodegradation rates and accentuate discrepancies in the removal of pharmaceuticals is the incubation time, which is usually arbitrary. The same compound can present different removal rates for different incubation times. Lastly, biodegradation rates can depend on the source, concentration, and pre-adaptation of the microbial inoculum. The removal efficiencies and lag times can be affected by these factors since the response of the microbial community will be different (Onesios et al., 2009). For instance, if the microbial community present in the inoculum has been previously exposed to pharmaceuticals, it may recognize more easily the compound, allowing a better adaptation of this community to the new conditions.

In summary, several intrinsic and extrinsic factors can affect biodegradation and biotransformation processes, both in natural environments and in engineered systems. More studies must be conducted to better understand and minimize the limitations that may arise in the development of bioremediation technologies. Despite the constraints that can appear, as in any other technologies, the low negative impacts of implementation, the need of no additional constructions for implementation, the high efficiency and the long-term viability (Shishir et al., 2019) make bioremediation technology as a sustainable solution that should be considered for the recovery of sites contaminated with pharmaceuticals and other pollutants.

1.8 General overview

Pharmaceutical compounds have a prevalent role in our society and their consumption tend to increase, since they are essential to face human and veterinary illnesses and to provide a better quality of life. With their continuous consumption, the incessant release of pharmaceuticals into the environment will be inevitable. After the improvement of detection methods, the next step to take is to find sustainable and efficient technologies to tackle this problem, both to prevent the environmental input of these compounds and to remove/recover impacted environments. Bioremediation technologies based on microbial communities with the capacity to degrade pharmaceuticals have been presented as a possible solution. Degradation of pollutants by microorganisms is known to be an important detoxification process in nature and it has been proved that sustainable technologies based on degrading microorganisms are a suitable solution to be developed and applied for the recovery of contaminated environments. To our best knowledge, bioremediation technology has not been applied for the removal of pharmaceuticals in natural environments, despite the increasing number of studies looking into the potential of microorganisms to metabolize/degrade pharmaceuticals.

Several studies have been performed involving bacterial communities or single strains able to degrade different pharmaceutical classes and proved to be an option to face pharmaceuticals contamination. However, more studies are needed regarding the development and application of bioremediation technology in different environments contaminated with these compounds. There are several topics regarding the development of this technology that should be addressed in future studies. For instance, tests in natural media should be performed to investigate the effects of the addition of bacterial formula in natural communities and to evaluate if the added microbial community continues to have a high removal efficiency. In natural media, there are several factors that are continually changing and that can be a step back in the development of this technology. For example, temperature, hydraulic conditions as other physical-chemical properties can influence the communities in the contaminated site and affect the performance of the designed technology. The concomitant presence of other pollutants (metals, nanoparticles, pesticides and other pharmaceuticals with different function and structure) should also be tested since their presence can inhibit the added bacteria or exert unexpected antagonist or synergetic effects influencing the performance of this technology. Other major aspect that should be addressed is the metabolites that are generated during the degradation process. Most of the metabolites that are formed are unknown, justifying more studies on the metabolic pathways and final degradation products. The goal of the biodegradation process is that the

generated metabolites have less toxicity or be completely inactive. For example, for halogenated pollutants, microbial dehalogenation is a crucial reaction since halogen atoms are usually responsible for the environmental recalcitrance of the molecule, also increasing the chances of generating less toxic metabolites (Janssen et al., 2001).

Most of the existing technologies are directed to WWTPs and there is a lack of technologies able to be applied in natural environments. Bioremediation technology can fulfill this gap, being a cost-effective technology that can be applied both *in situ* and *ex situ*. By using natural communities, this technology can be applied to restore natural ecosystems like estuarine areas and rivers, as well in WWTPs to avoid the release of pharmaceuticals into the environment. In the latter, this technology can help improving the biological treatment, maintaining its main goal of removing organic matter and nutrients while exploring and enhancing the bacteria that can also degrade pharmaceuticals. By analyzing the bacteria present in the biological reactor it is possible to select those with better skills for the degradation of pharmaceutical compounds, develop a bacterial cocktail, and bioaugment with it the biological reactor without compromising the degradation of the bulk organic matter. However, this can be difficult to develop since different wastewater facilities may have different biological treatments and the microbial community of the biological treatment can change due to the type of influent, season condition and WWTP configuration. Thus, to obviate this problem, bioremediation could be applied in a tertiary treatment, after the biological treatment, although, a tertiary treatment requests space. Both options present challenges that should be addressed in future studies. At last, another aspect that should be studied in the future is the nutritional status of the contaminated site. This is a very important issue since the amount of nutrients in the system is a limiting factor for bacterial growth and degradation of pharmaceuticals. To avoid eutrophication or nutrient depletion, an optimal C: N: P (Carbon, Nitrogen and Phosphorous) ratio must be ensured. In the same way, to allow the growth and survival of the bioaugmentation formula in the contaminated environment, a proper amount of nutrients should be available in the matrix. This amount should be directly correlated with the concentration of bacteria to be added to the environment, which in turn should be directly correlated with the concentration of the contaminant (*i.e.*, the amount of carbon source) in the affected site. Therefore, this is also an aspect that should be explored in the development of the bioremediation technology.

Despite the various issues that need further investigation, bioremediation remains a promising solution to prevent emissions of pharmaceutical products to the environment or to remediate ecosystems impacted by these compounds. In this chapter, it was showed that microbial enrichment processes allow to obtain bacteria capable of effectively degrading

different classes of pharmaceuticals. However, the use of microorganisms not isolated from the affected site can cause negative and undesired impacts or result in low removal efficiencies of the target compound. Introducing exogenous microorganisms in the environment can disrupt and affect the dynamics of the natural community and the functioning of the ecosystem. To overcome this issue, microbial enrichments should be carried out with autochthonous microorganisms recovered from the affected site. In doing so, the impacts in the natural community can be diminished. Moreover, this microbial community may have less constraints in adapting to the contaminated environment and exhibit a better performance. As such, the development of bioremediation technologies should focus on the potential of native degrading communities, to ensure a better and more sustainable solution to tackle environmental contamination by pharmaceutical compounds.

1.9 Aims and general structure of the PhD Thesis

This work aimed to explore the potential of native microorganisms to biodegrade different pharmaceuticals, for the development of clean-up technologies to recover contaminated environments. Five microbial cultures previously enriched with paroxetine or bezafibrate were obtained from an estuarine sediment and activated sludge inoculum, under static and agitated conditions (Duarte et al., 2019).

This thesis is structured in 5 major chapters. In chapter 1, a general introduction regarding the occurrence and fate of pharmaceuticals as well as an overview focused on the bioremediation technology is presented.

In chapter 2, isolation and identification the culturable bacteria from the five enriched cultures was performed. In addition, the capacity of consortia assembled with the isolated bacterial strains to biodegrade the target pharmaceuticals was evaluated. In this chapter, is unveiled the taxonomic identification of each bacterial isolate and the potential of the assembled consortia to degrade the respective pharmaceutical.

In chapter 3, optimization of two degrading bacterial consortia (selected based on the degrading capacity observed in chapter 2), for the degradation of paroxetine and bezafibrate. For that, the potential of each bacterial strain to degrade the respective pharmaceutical was accessed in biodegradation experiments, either as a single strain or as a consortium. Moreover, the versatility of each bacterial consortium to bioremediate the other pharmaceutical was also evaluated, by exposing the paroxetine degrading consortium to bezafibrate and bezafibrate degrading consortium to paroxetine.

In chapter 4, it was evaluated the potential of native microorganisms (selected based on the degrading capacity observed in chapter 3) to degrade paroxetine in natural media experiments. Furthermore, the effects of bioaugmentation process, with native microorganisms, on the dynamics of estuarine natural community was assessed, through the amplification and the sequencing of (V4-V5) 16S rRNA gene fragment by Illumina MiSeq platform.

In chapter 5, a general discussion was performed to connect the different experiments performed in this PhD thesis and the main conclusions from retrieved from this work.

Chapter 2

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Potential of bacterial consortia obtained from different environments for bioremediation of paroxetine and bezafibrate

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2.1 Abstract

This work aimed to isolate and identify the culturable bacterial strains from five enriched cultures and investigate the capability of these strains to biodegrade paroxetine or bezafibrate, when assembled into consortia. Bacterial strains were isolated from cultures enriched with paroxetine and bezafibrate using, as microbial inocula, an estuarine sediment and activated sludge from an associated wastewater treatment plant. All the isolated strains were identified through 16S rRNA gene sequencing. Pharmaceutical compounds were analyzed by HPLC-DAD (diode array detector). Fluoride release was analyzed using a fluoride ion-selective electrode to evaluate the extent of paroxetine defluorination. High removal efficiency of pharmaceuticals (>97%) was observed for three of the five assembled consortia. A total of 28 bacterial strains were isolated from an activated sludge enrichment and 20 bacterial strains were isolated from an estuarine sediment enrichment. The genera *Pseudomonas* and *Acinetobacter* were the most dominant in the consortia derived from activated sludge, while the genus *Pseudomonas* was dominant in the consortia derived from estuarine sediment. This work highlights the potential of native bacterial consortia obtained from different environmental sources to biodegrade paroxetine or bezafibrate, and unveils the taxonomic diversity associated to the biodegradation of these compounds. These consortia may be considered in the future for the development of new bioremediation tools for environmental restoration.

2.2 Introduction

The exponential development of society led in the last years to an increased consumption of pharmaceuticals to treat several diseases. However, the presence of pharmaceuticals in the environment and their associated effects are triggering a growing public concern, being one of the most relevant topics in environmental research today. For most pharmaceuticals, no regulation has been implemented yet concerning this issue, and there is still a lack of knowledge about their transformation pathways, their effects on aquatic organisms, and also concerning their persistence or degradability rates in the environment (Gauthier et al., 2010; Klatte et al., 2017).

Antibiotics, anti-inflammatory drugs, antidepressants, analgesics and lipid regulators are among the most prescribed pharmaceuticals worldwide. These compounds can be excreted and enter into the environment in the parental form (since most of them are not fully metabolized by human or animal body) or as metabolites (Rivera-Utrilla et al., 2013; Żur et al., 2018). Pharmaceuticals can enter in the environment through direct or indirect sources such as WWTPs (from urban agglomerates, hospitals, aquaculture, livestock and pharmaceutical manufacture industry), illegal discharges of untreated effluents, improper disposal of unused or expired pharmaceuticals, manure and sludge contaminated with pharmaceuticals when used as organic fertilizers and through leachates from landfills (Eslami et al., 2015; Madureira et al., 2010; Togunde et al., 2012; Wellington et al., 2013). Concentrations of pharmaceuticals ranging between ng L^{-1} and mg L^{-1} have been widely reported in surface water (Gros et al., 2012; Osorio et al., 2016), groundwater (López-Serna et al., 2013; Petrović et al., 2014), drinking water (Aristizabal-Ciro et al., 2017), seawater (Gros et al., 2012), wastewaters and sludge (Ekpeghere et al., 2017; Rivera-Jaimes et al., 2018) and sediments and soils (Biel-Maeso et al., 2017; Vazquez-Roig et al., 2012).

Once in the environment, the fate of pharmaceuticals is governed by abiotic (photolysis, hydrolysis), physical (dilution, diffusion or transport) and biotic processes. Most pharmaceuticals have complex structures with diverse functional groups (e.g., halogens, sulfate), which makes their degradation challenging (Żur et al., 2018).

It is consensual that effective methods are needed to remove these pollutants from the environment. Several technologies have emerged to tackle this problem, like advanced oxidation processes (AOPs), membrane filtration processes, adsorption to granular activated carbon (GAC), biological activated carbon (BAC), powdered activated carbon (PAC), nanofiltration and reverse osmosis (Luo et al., 2014; Yang et al., 2017). Most of these techniques were designed for application in wastewater treatment plants because

their effluents are one of the main inputs of pharmaceuticals in the environment. However, there are other inputs of pharmaceuticals that contribute to the contamination of the environment, which need to be remediated. Thus, there is a need for technologies that can be applied in the natural environment, like estuarine areas, freshwaters, sediments, among others. Bioremediation, a technology based on the use of microorganisms to degrade different pollutants, can be a sustainable and cost-effective alternative for removal of pollutants in natural environment. In fact, degradation of pollutants by autochthonous microorganisms is one of the most important removal processes in impacted environments, which can lead to partial or entire elimination of pollutants through metabolic and/or co-metabolic pathways (Caracciolo et al., 2015). These microorganisms can use organic pollutants, like pharmaceuticals, both as a primary substrate or as a co-substrate (Müller et al., 2013; Onesios et al., 2009). Therefore, one should focus on these specific degrading microorganisms and find new technologies for stimulation of their activities and future use, taking advantage of the natural processes already occurring in the environment. The use of microbial consortia, instead of individual pollutant-degrading microorganisms, can be an advantageous strategy, as the cooperative interaction or synergistic effects of different bacterial strains can play a crucial role in the degradation of the target compound(s) (Aissaoui et al., 2017; Sepehri et al., 2020; Sepehri & Sarrafzadeh, 2018; Zhao et al., 2015). Some bioremediation technologies using non-native microorganisms already exist. However, their adaptation in the affected site can be difficult or can lead to a disruption of the existing community, which may cause an imbalance in the ecosystem. Therefore, bioremediation with autochthonous microorganisms arises as a more suitable approach, where microbial communities native from the impacted environment are used for specific pollutants removal/degradation. Application of autochthonous bioremediation in natural environments for the removal of pharmaceuticals is still unexplored and its potential should be evaluated given the high relevance of removing these compounds from the environment.

The pharmaceuticals paroxetine and bezafibrate are representatives of two relevant pharmaceutical families, namely antidepressants and lipid-lowering agents. Both pharmaceuticals are halogenated compounds and this property makes them more stable and resilient to degradation. Paroxetine is a selective serotonin reuptake inhibitor (SSRI) antidepressant, widely prescribed to treat anxiety problems and depression (Cunningham et al., 2004), and has been detected in underground waters (5.17 – 30.2 ng L^{-1}) (López-Serna et al., 2013), surface waters (0.27 – 40 ng L^{-1}) (Gros et al., 2012; Osorio et al., 2016) and sediments (0.05 – 0.76 ng g^{-1}) (Osorio et al., 2016). Bezafibrate is a lipid-lowering agent frequently prescribed to decrease blood levels of cholesterol and triglycerides (Monk

& Todd, 1987), and has been detected in surface waters, at concentrations ranging between 0.84 – 13.40 ng L^{-1} (Reis-Santos et al., 2018). Effects of both pharmaceuticals in non-target organisms were already described. Velasco-Santamaría et al. reported that concentrations up to 200 µg L^{-1} of bezafibrate can cause physiological effect on male zebrafish and can alter expression patterns of genes involved in the gonadal steroidogenesis and led also to changes in the spermatogenesis (Velasco-Santamaría et al., 2011). Negative immunological effects on *Mytilus edulis* were reported by Lacaze et al., where cytotoxicity, immunotoxicity and genotoxicity in mussel hemocytes were observed due to paroxetine exposure (Lacaze et al., 2015).

In a previous study, we obtained five enriched cultures capable of efficiently biodegrading paroxetine and bezafibrate, using sediments from the Douro river estuary (North of Portugal) and activated sludge from an associated WWTP as microbial inocula (Duarte et al., 2019). In the present study, the main goal was to isolate and identify the culturable bacteria from these enriched cultures and evaluate the capacity of consortia assembled with the isolated bacterial strains to biodegrade the target pharmaceuticals. This study aims to reveal for the first time the diversity and phylogeny of microorganisms associated with the biodegradation of paroxetine and bezafibrate and evaluate their efficiency to biodegrade these compounds when assembled in a consortium.

2.3 Material and methods

2.3.1 Reagents

Bezafibrate and paroxetine HCl were acquired from Sigma-Aldrich and Enzo Life Sciences, respectively. HPLC grade methanol, acetonitrile and formic acid (98%) were acquired from Sigma-Aldrich. Paroxetine and bezafibrate solutions were prepared by dissolving a known quantity of each compound in methanol.

All the materials used in this study were decontaminated and/or sterilized to avoid chemical and/or biological contamination. Biological sterilization was performed by autoclaving the materials (120°C, 20min), whereas chemical decontamination was carried out by emerging the materials in a chloride acid bath for 24h (10%, v/v), followed by washing with deionized water and drying at 40°C.

2.3.2 Isolation of bacterial strains from cultures enriched with paroxetine and bezafibrate

The bacterial strains used in the present study were isolated from a previous enrichment experiment with paroxetine and bezafibrate (Duarte et al., 2019). In Duarte et al. (Duarte et al., 2019), estuarine sediment collected in Douro river estuary or activated sludge from an associated WWTP were used to inoculate a mineral-salts medium (MM) (full constitution in supplementary material SM 1) (Alexandrino et al., 2018) doped with 1 mg L^{-1} of bezafibrate or paroxetine and 500 mg L^{-1} of sodium acetate as a co-substrate. The enriched cultures were incubated under two operational conditions: agitated (130 rpm) or static, during seven feeding cycles of two weeks (14 weeks in total) (Duarte et al., 2019). Five enriched cultures showing removal efficiencies of the target pharmaceuticals higher than 97% (Duarte et al., 2019) were selected for the present study, namely two cultures enriched with paroxetine and three enriched with bezafibrate. These enriched cultures were selected considering not only their removal efficiencies but also the time that they needed to reach the highest degradation. The enriched cultures in static conditions were selected as they displayed better removal in a shorter enrichment period and, in the case of paroxetine, also higher defluorination rates. In addition, one enriched culture derived from activated sludge inoculum, in agitation, was selected since activated sludge systems are in constant agitation. The consortium exposed to bezafibrate under agitation was selected for this study as it was the one that was able to achieve a consistence removal efficiency above 97%. On the contrary, the consortium exposed to paroxetine under agitation was not selected since it displayed low defluorination percentages (<50%) during most of the cycles (Duarte et al., 2019).

In the present study, isolation and identification of bacterial strains in those enriched cultures was performed (Fig. 3). For that, the enriched cultures obtained in the study of Duarte et al. (Duarte et al., 2019) were spread in several tenfold dilutions (up to 10^{-5}) onto plate count agar (PCA) and MM agar plates supplemented with 1 mg L^{-1} of bezafibrate or paroxetine and 500 mg L^{-1} of sodium acetate. After 48h of incubation at 28°C, bacterial colonies with different morphologies were visually identified. These colonies were purified by repetitive streaking in the same agar plates where they were spotted. The purified strains were preserved in 25% glycerol at -80°C.

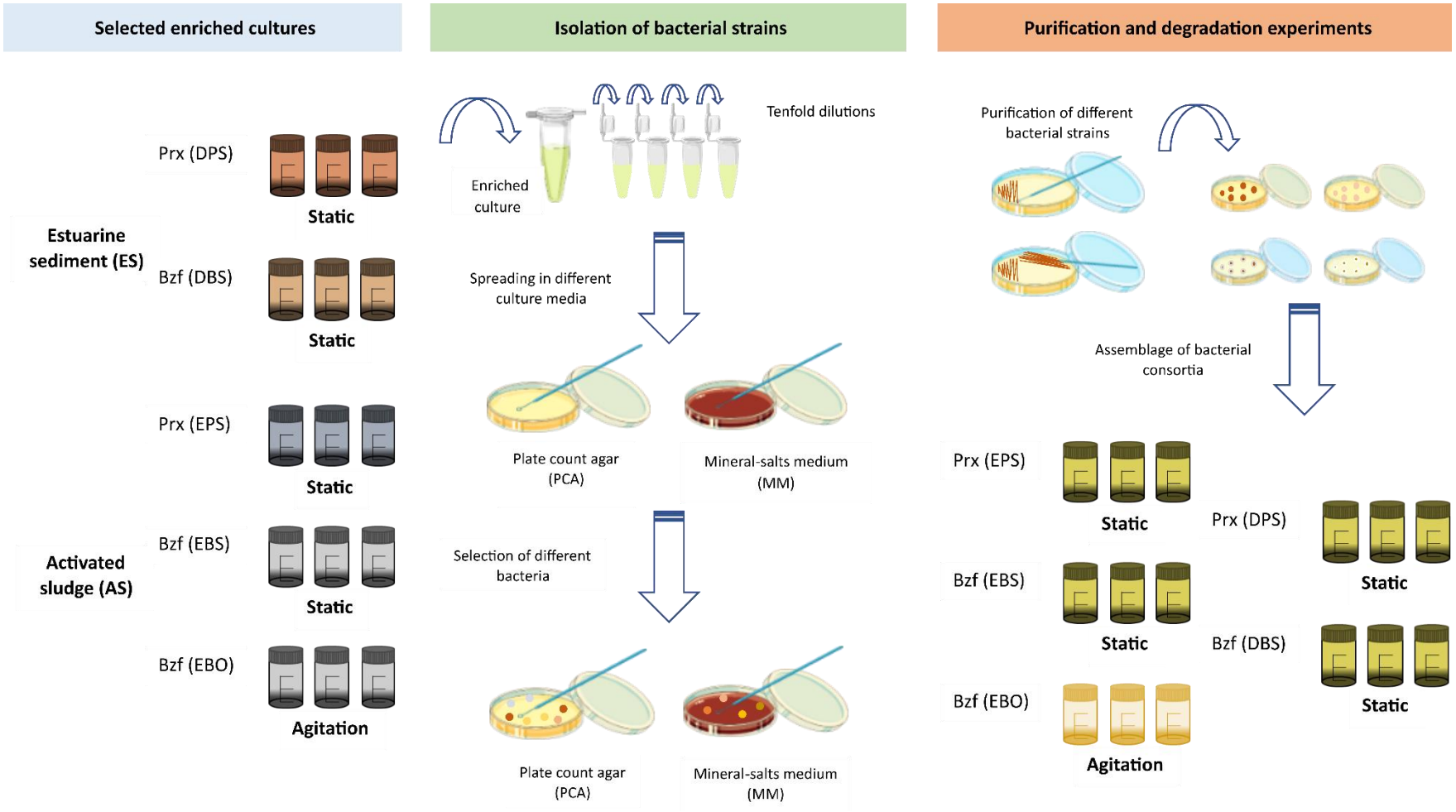

Figure 3 - Experimental design for the isolation and purification of bacterial strains from the enriched consortia and assemblage of biodegradation experiments. Bzf – bezafibrate; Prx – Paroxetine. DPS- estuarine sediment under static conditions, exposed to paroxetine; DBS - estuarine sediment under static conditions, exposed to bezafibrate; EPS- activated sludge under static conditions, exposed to paroxetine; EBS – activated sludge under static conditions, exposed to bezafibrate; EBO – activated sludge under agitated conditions, exposed to bezafibrate ; AS – activated sludge. PCA – plate count Agar; MM – mineral-salts medium.

2.3.3 Taxonomic identification of the purified bacterial strains

The bacterial isolates were identified through 16S rRNA gene sequence analysis. DNA from each isolate was extracted using the E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA), according to the protocol provided by the supplier. Then, hypervariable regions V1–V9 of the 16S rRNA gene were amplified using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991). Specifically, a polymerase chain reaction (PCR) was carried out in aliquots of 10 µL containing between 1-3 µL of DNA template, 1 µL of each primer (2 mM) and 5 µL of QIAGEN Multiplex PCR Kit (Qiagen, Hilden, Germany). Additionally, sterile water was added to the samples with 1 µL of DNA template to complete reaction volume. PCR reaction program started with initial denaturation at 95°C for 15 min, followed by 30 cycles at 94°C for 30 s, 48°C for 90 s, and 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were visualized in a 1.5% electrophoresis agarose gel and quantified using Qubit® dsDNA HS Assay Kit and Qubit 3.0 fluorometer (Invitrogen), according to the protocol provided by the supplier. Purification and sequencing of the amplicons was performed by GenCore, I3S (Instituto de Investigação e Inovação em Saúde), in Porto, Portugal. The raw sequences provided by I3S were analyzed using Geneious 11.1.4 software (Biomatters Ltd, Auckland, New Zealand) and the consensus sequences were submitted to GenBank for taxonomic identification (Nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi); both nucleotide collection and 16S ribosomal RNA sequences were used). To ensure and compare the results, EzBioCloud (Yoon et al., 2017) and Ribosomal Database Project (RDP) (Cole et al., 2014) databases were also used.

Phylogenetic trees were built using Maximum Likelihood method with 1000 bootstraps with the software Molecular Evolutionary Genetics Analysis (MEGA) version 7.0.26 (Kumar et al., 2016). For that, the closest sequences (type strains) on the GenBank database at National Center for Biotechnology Information (NCBI) (16S ribosomal RNA sequences (Bacteria and Archaea)) were selected for each isolate and aligned with the consensus sequences using MUSCLE from the Geneious software package. The most suitable nucleotide evolutionary model was calculated based on the lowest Akaike Information Criterion (AIC) using MEGA 7.0.26 software.

2.3.4 Biodegradation of paroxetine and bezafibrate by bacterial consortia assembled with isolated strains

The isolated bacterial strains (Tables 1 and 2, see section 2.4.1) were assembled into 5 consortia, according to the enriched culture from which they were derived (Figure 3). The isolates were grown on PCA or MM agar plates and five different consortia were prepared for the biodegradation experiments, by suspending in MM equal proportions of the appropriate bacterial strains. These inocula were then used to inoculate 250 mL Schott flasks containing 40 mL of MM to an optical density (OD) of ca. 0.5. The consortia were doped with 1 mg L^{-1} of bezafibrate or paroxetine and 500 mg L^{-1} of sodium acetate and incubated in dark conditions, at room temperature (21ºC) and under static or agitation conditions (orbital agitation, 130 rpm). (Tables 1 and 2, see section 2.4.1). Each consortium was tested in triplicate. The experiment was conducted for three feeding cycles of two weeks each. In each cycle, consortia were fed twice a week with 500 mg L^{-1} of sodium acetate to promote metabolic activity and bacterial co-metabolism, and depletion of oxygen in the flasks was avoided by transferring the cultures once a week to new sterilized flasks. At the beginning of a new feeding cycle, 20 mL of each consortium was transferred to a new sterilized flask containing 20 mL of MM doped with 1 mg L^{-1} of paroxetine or bezafibrate and 500 mg L^{-1} of sodium acetate. The remaining culture was collected to analyze removal of the pharmaceuticals from the culture medium, microbial growth and fluoride ion release (only for paroxetine).

2.3.5 Analytical methods

2.3.5.1 Analysis of the concentration of paroxetine and bezafibrate in the culture medium

The concentration of the target pharmaceuticals in the culture medium was analyzed at the beginning and at the end of each feeding cycle by HPLC – DAD. Samples from each consortium were collected and transferred into amber glass vials and centrifuged at 13000 rpm for 15 min (VWR MICROSTAR 17, VWR International, USA). The supernatant was analyzed in a HPLC Beckam Coulter equipment (System Gold) equipped with a diode array detector (module 168) and an automatic sampler (module 508), using a gradient of two solvents (water/formic acid (99:1, v/v) and acetonitrile, both previously degassed in an ultrasonic bath) as described in Duarte et al. (Duarte et al., 2019). Quantification of each compound was obtained through external calibration with aqueous standard solutions (in MM), ranging between 0.1 to 2 mg L^{-1} of each pharmaceutical.

2.3.5.2 Analysis of fluoride ion release

For the determination of fluoride anion concentration in the consortia doped with paroxetine, samples collected at the beginning and at the end of each feeding cycle were centrifuged at 13000 rpm for 15 min (VWR MICROSTAR 17, VWR International, USA). The resulting supernatant was then analyzed using a fluoride ion-selective electrode (Crison 9655 C, Crison Instruments, S.A., Spain). For calibration, aqueous standard solutions of sodium fluoride, ranging between 0.001 to 1 mM, were prepared in MM. To minimize interferences, a total ionic strength adjustment buffer (TISAB III) was added to all samples and standards in a 1:10 ratio.

2.3.6 Statistical analysis

For all samples, triplicates were analyzed and treated independently, and the mean values and respective standard deviations were calculated. Statistical tests were performed using the software STATISTICA version 12 (StatSoft, Inc., 2013). For the parameters removal efficiency of paroxetine or bezafibrate and fluoride ion release, significant differences among samples were evaluated through a parametric Student's t-test, using mean values and corresponding standard deviations of the replicates (n=3). Significant differences were assumed for p-value below or equal to 0.05 (confidence level of 95%).

2.4 Results

2.4.1 Bacterial composition of the consortia

A total of 48 bacterial strains were isolated from the five cultures enriched with paroxetine or bezafibrate, selected for this study (Tables 1 and 2). To recover as many bacterial isolates as possible from the enriched cultures, two culture media were used: PCA, a non-selective medium, and MM supplemented with the target pharmaceuticals, the same culture medium used in the enrichment process. Thirty-four isolates were retrieved from PCA agar plates, while 14 bacterial strains were recovered from MM. The number of isolates obtained from each enriched culture varied between 8 and 11 (Tables 1 and 2).

Taxonomic identification of the bacterial isolates through 16S rRNA gene sequencing revealed that most isolates were affiliated with the phylum Proteobacteria (Figure 4). Two other phyla were also identified in the isolates obtained from estuarine sediment, Actinobacteria and Bacteroidetes. Some isolates derived from activated sludge were also affiliated with the latter phylum (Figure 4).

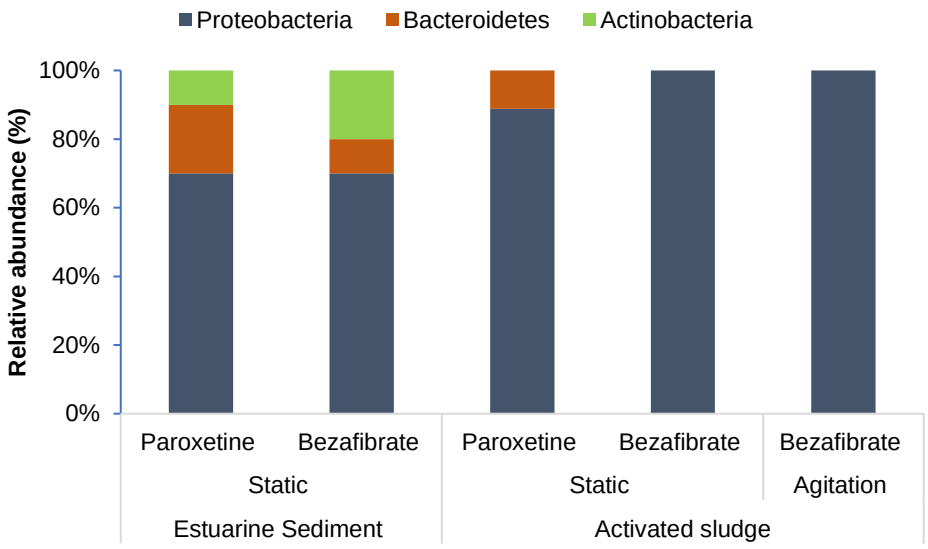

Figure 4 - Relative abundance of bacterial phyla within the different cultures pre-enriched with paroxetine and bezafibrate selected for this study.

In terms of genera, the genus *Pseudomonas* was the most predominant in all enriched cultures (Figure 5 and Tables 1 and 2). The genus *Acinetobacter* sp. was present in all enriched cultures obtained in static conditions. In addition, the genera *Shewanella* and *Leadbetterella* were detected only in cultures exposed to paroxetine. The genus *Hydrogenophaga* was detected in both cultures obtained from activated sludge inoculum in static conditions whereas the genus *Microbacterium* was only detected in the enriched cultures derived from estuarine sediment, also in static conditions. In general, in all enriched cultures, it was possible to recover several strains belonging to a different genus, except the one enriched from activated sludge with bezafibrate under static conditions, where only 3 genera were recovered (Figure 5). Furthermore, in the case of cultures obtained from activated sludge exposed to bezafibrate, different incubation conditions selected different degrading bacterial genera.

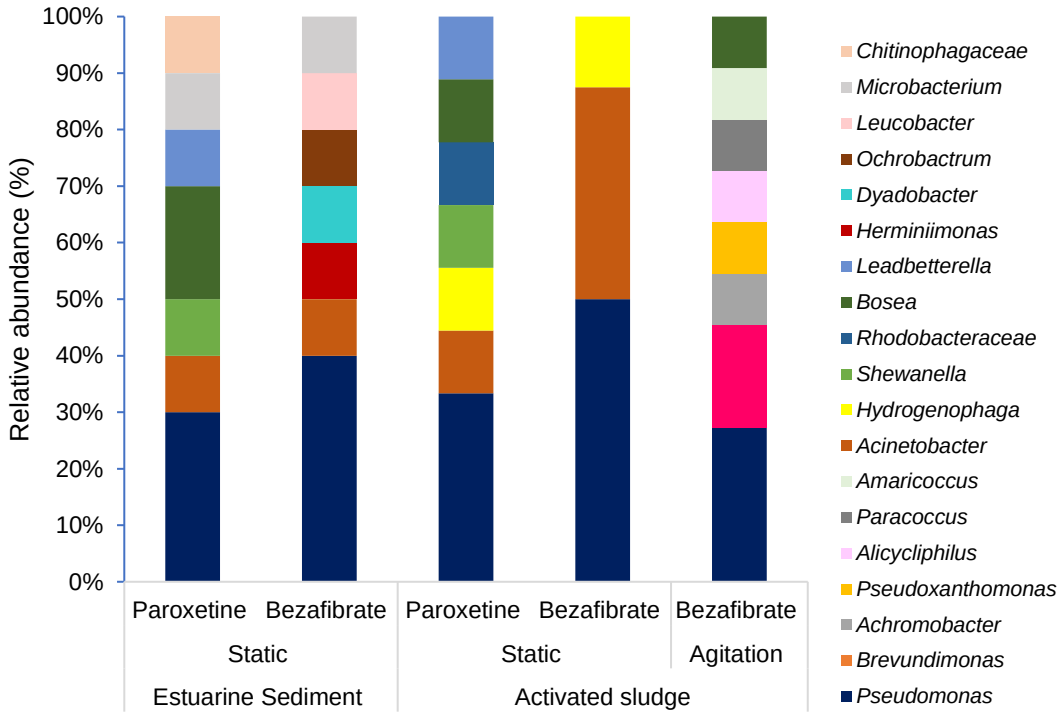

Figure 5 - Relative abundance in terms of bacterial genera (except for Chitinophagaceae affiliates and Rhodobacteraceae affiliates for which only family was obtained) of the strains isolated from the cultures pre-enriched with paroxetine and bezafibrate selected for this study.

Taxonomic analysis and subsequent phylogenetic studies revealed that a number of the isolates obtained from the enriched cultures represent potential novel taxonomic entities, as the similarity of their sequences to the closest strains presented in the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database showed values below 98.7% (Stackebrandt & Ebers, 2006). This is the case for strains DPS 4 and DPS 8, affiliated with the phylum Bacteroidetes and isolated from the consortium pre-enriched from estuarine sediment with paroxetine under static conditions (Figure 6); strains EPS 6, EPS 8 and EPS 10, integrating the phyla Proteobacteria and Bacteroidetes, respectively, and obtained from the consortium pre-enriched from activated sludge with paroxetine under static conditions (Figures 6 and 7); strains DBS 3 and DBS 5, belonging to the phyla Bacteroidetes and Actinobacteria, respectively, and isolated from the consortium pre-enriched from estuarine sediment with bezafibrate under static conditions (Figures 6 and 7); strains EBS 6 and EBS 9, both affiliated with the phylum Proteobacteria, and isolated from the consortium pre-enriched from activated sludge with bezafibrate under static conditions (Figures 6 and 8); and strains EBO8 and EBO11, affiliated with the phylum Proteobacteria and derived from the consortium pre-enriched from activated sludge with bezafibrate under agitation

conditions (Figure 7). In addition, the strain DPS 4 can represent a potential new genus since this strain displayed identity values below 94.5% (Table 1) (Yarza et al., 2014).

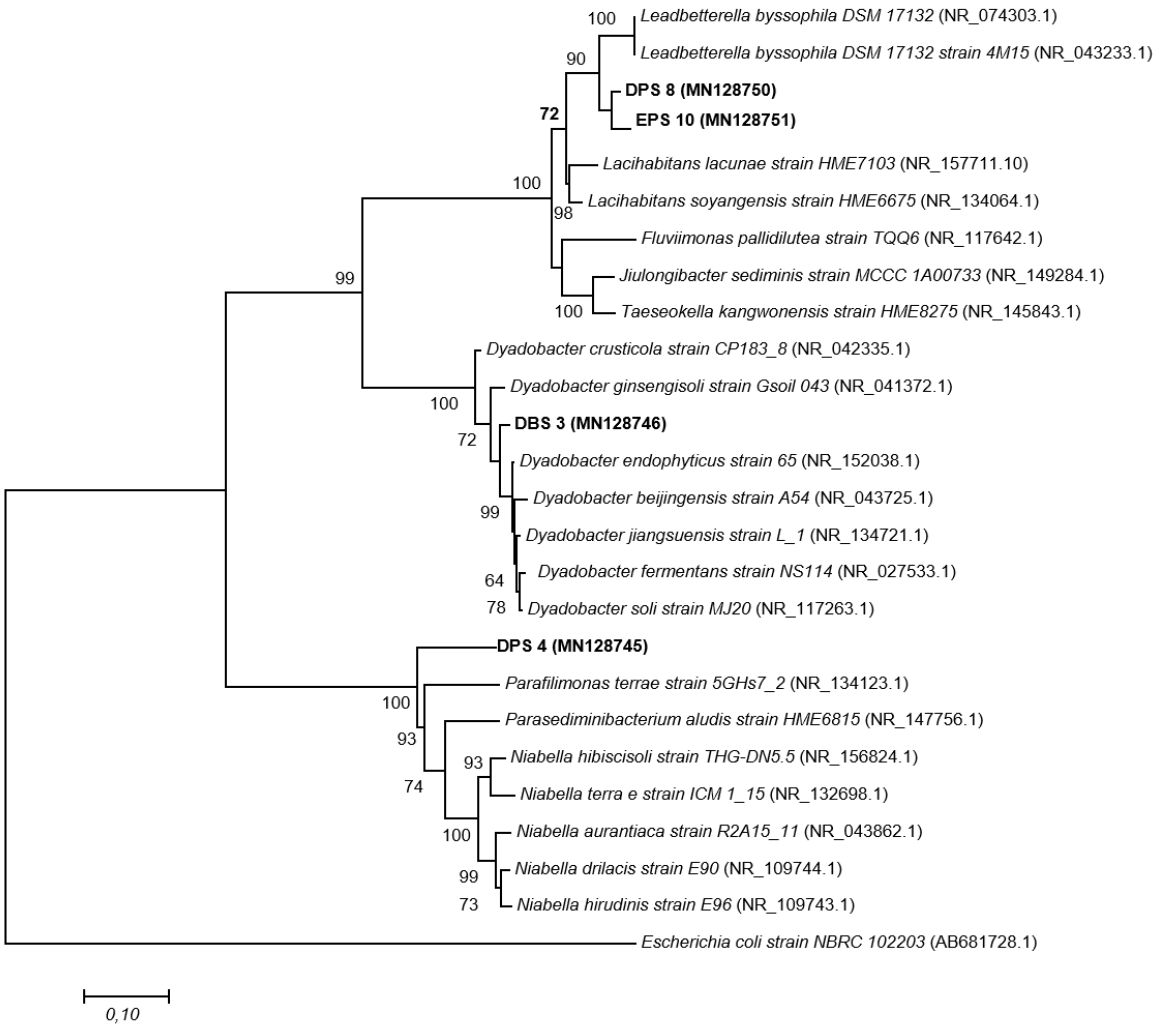

Figure 6 - Phylogenetic tree of four potential new species belonging to the phylum Bacteroidetes, isolated from estuarine sediment enriched cultures or activated sludge enriched cultures, based on 16S rRNA gene homology with their nearest strains (26 sequences, with a final alignment with 1394 bp). Numbers at nodes represent bootstrap values when higher than 60%. Numbers in each branch corresponds to GenBank accession numbers. Phylogenetic tree built based on the Maximum Likelihood test, with Hasegawa-Kishino-Yano (HKY + G + I) nucleotide evolutionary model.

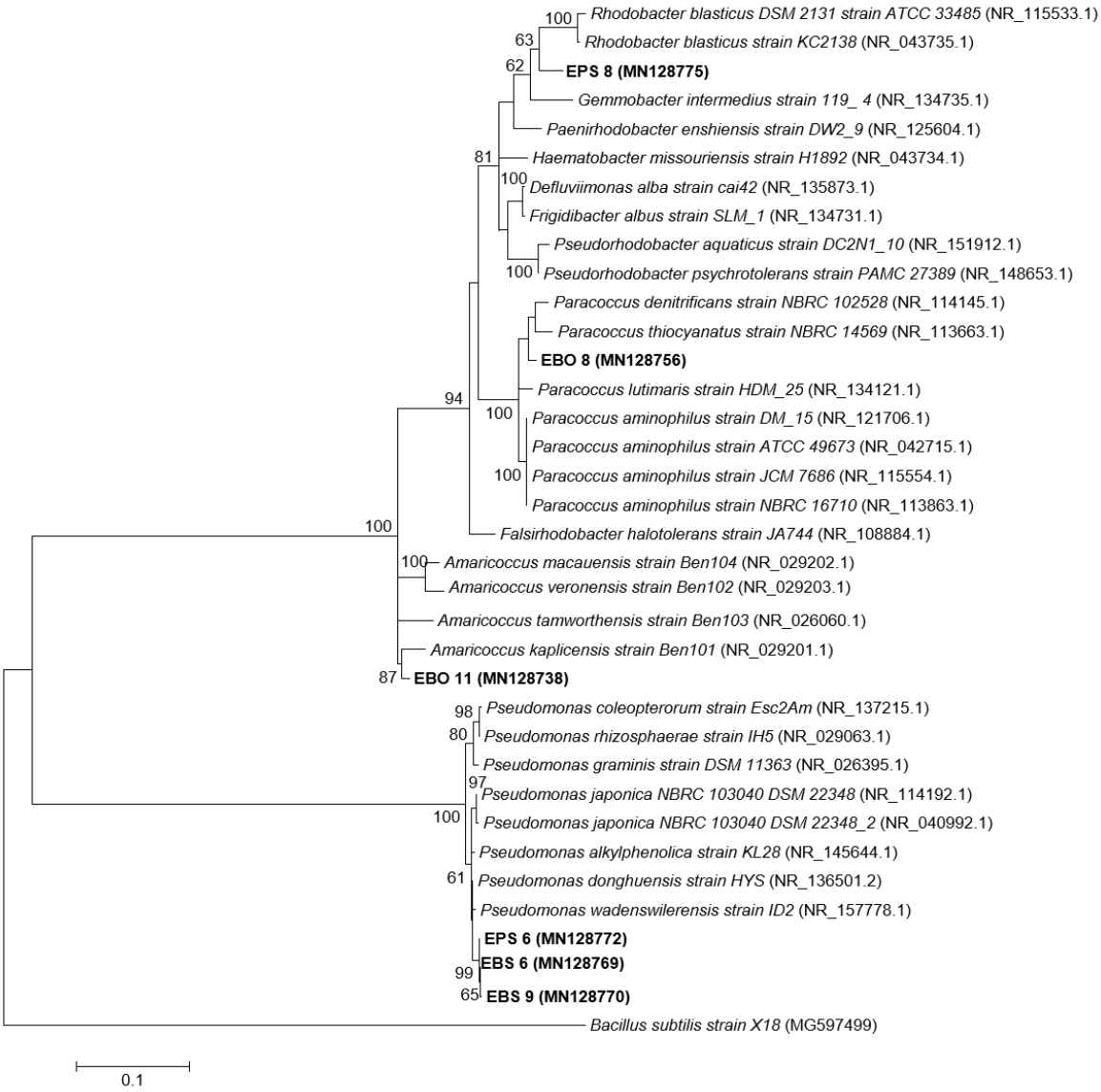

Figure 7 - Phylogenetic tree of two potential new species belonging to the phylum Proteobacteria, isolated from activated sludge enriched cultures, based on 16S rRNA gene homology with their nearest strains (36 sequences, with a final alignment with 1391 bp). Numbers at nodes represent bootstrap values when higher than 60%. Numbers in each branch correspond to GenBank accession numbers. Phylogenetic tree built based on the Maximum Likelihood test, with Tamura 3 parameter (T92 + G + I) nucleotide evolutionary model.

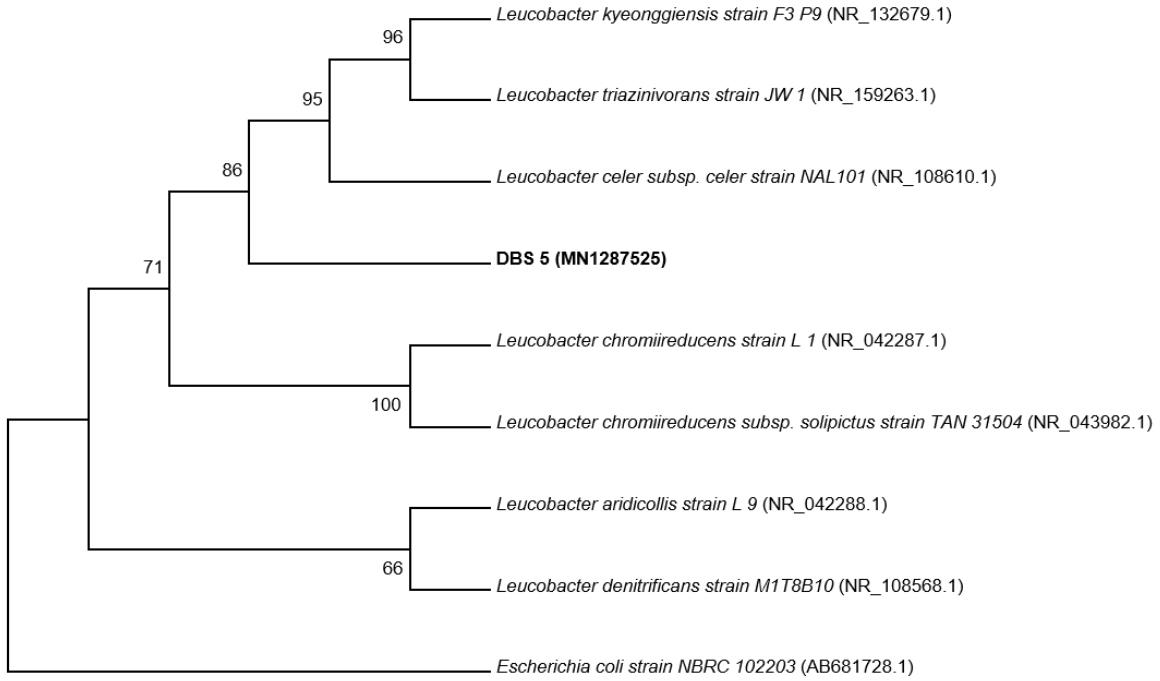

Figure 8 - Phylogenetic tree of one potential new species belonging to the phylum Actinobacteria, isolated from estuarine sediment enriched cultures, based on 16S rRNA gene homology with their nearest strains (9 sequences, with a final alignment with 1436 bp). Numbers at nodes represent bootstrap values when higher than 60%. Numbers in each branch correspond to GenBank accession numbers. Phylogenetic tree built based on the Maximum Likelihood test, with Kimura 2 parameter (K2 + G + I) nucleotide evolutionary model.

Table 1 - Taxonomic identification of bacterial isolates obtained from the cultures pre-enriched with paroxetine, using estuarine sediment (Douro river estuary) and activated sludge from an associated WWTP as inocula. Strains isolated from estuarine sediment under static incubation were named as DPS, while isolates retrieved from activated sludge, also under static incubation, were named as EPS. Bp – base pairs; PCA –Plate Count Agar; MM - Mineral-salts Medium. * potential new species according to the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database.

Inoculum	Incubation condition	Microbial strains isolated	Isolation medium	Taxonomic Identification		Query Coverage (%)	Pairwise Identity (%)	Sequence Length (bp)	GenBank accession number
				Closest relative	Phylum				
Estuarine sediment	Static	DPS 1	MM with Paroxetine	*Pseudomonas* sp.	Proteobacteria	100	98.79	1402	MN128762
		DPS 2	MM with Paroxetine	*Bosea* sp.	Proteobacteria	100	99.70	1335	MN128739
		DPS 3	PCA	*Shewanella* sp.	Proteobacteria	100	99.29	1412	MN128776
		DPS 4*	PCA	*Chitinophagaceae bacterium*	Bacteroidetes	97	88.85	1367	MN128745
		DPS 5	PCA	*Acinetobacter* sp.	Proteobacteria	100	99.15	1401	MN128733
		DPS 6	PCA	*Pseudomonas* sp.	Proteobacteria	100	98.72	1399	MN128764
		DPS 7	PCA	*Bosea* sp.	Proteobacteria	100	99.63	1343	MN128740
		DPS 8*	PCA	*Leadbetterella* sp.	Bacteroidetes	100	95.55	1365	MN128750
		DPS 9	PCA	*Microbacterium oxydans*	Actinobacteria	100	99.86	1389	MN128754
		DPS 10	MM with Paroxetine	*Pseudomonas* sp.	Proteobacteria	100	99.29	1402	MN128763
Activated Sludge	Static	EPS 1	MM with Paroxetine	*Acinetobacter* sp.	Proteobacteria	99	99.15	1406	MN128737
		EPS 2	MM with Paroxetine	*Pseudomonas* sp.	Proteobacteria	100	99.80	990	MN128771
		EPS 4	PCA	*Shewanella* sp.	Proteobacteria	100	99.36	1414	MN128777

Activated Sludge	Static	EPS 5	PCA	*Hydrogenophaga* sp.	Proteobacteria	99	99.86	1396	MN128749
		EPS 6*	PCA	*Pseudomonas* sp.	Proteobacteria	99	98.65	1408	MN128772
		EPS 7	PCA	*Pseudomonas* sp.	Proteobacteria	100	98.79	1402	MN128773
		EPS 8*	PCA	*Rhodobacteraceae bacterium*	Proteobacteria	100	95.36	1332	MN128775
		EPS 9	PCA	*Bosea* sp.	Proteobacteria	100	99.63	1336	MN128742
		EPS 10*	PCA	*Leadbetterella* sp.	Bacteroidetes	99	94.81	1368	MN128751

Table 2 - Taxonomic identification of bacterial isolates obtained from the cultures pre-enriched with bezafibrate using estuarine sediment (Douro river estuary) and activated sludge from an associated WWTP as inocula. Strains isolated from estuarine sediment under static incubation were named as DBS; isolates retrieved from activated sludge, also under static incubation, were named as EBS and isolates obtained from activated sludge under agitation incubation were named as EBO. Bp – base pairs; PCA –Plate Count Agar; MM - Mineral-salts Medium. * potential new species according to the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database.

Inoculum	Incubation condition	Microbial strains isolated	Isolation medium	Taxonomic Identification		Query Coverage (%)	Pairwise Identity (%)	Sequence Length (bp)	GenBank accession number
				Closest relative	Phylum				
Estuarine sediment	Static	DBS 1	MM with Bezafibrate	*Acinetobacter* sp.	Proteobacteria	100	99.85	1364	MN128732
		DBS 2	MM with Bezafibrate	*Herminiimonas* sp.	Proteobacteria	100	99.93	1398	MN128747
		DBS 3*	PCA	*Dyadobacter* sp.	Bacteroidetes	100	97.38	1372	MN128746
		DBS 4	PCA	*Ochrobactrum rhizosphaerae*	Proteobacteria	100	99.33	1347	MN128755
		DBS 5*	PCA	*Leucobacter* sp.	Actinobacteria	99	97.13	1393	MN128752
		DBS 6	PCA	*Pseudomonas* sp.	Proteobacteria	100	99.86	1403	MN128758
		DBS 7	MM with Bezafibrate	*Pseudomonas* sp.	Proteobacteria	100	99.77	1328	MN128759
		DBS 8	MM with Bezafibrate	*Pseudomonas* sp.	Proteobacteria	100	99.57	1403	MN128760
		DBS 9	PCA	*Pseudomonas* sp.	Proteobacteria	100	99.71	1402	MN128761
		DBS 10	PCA	*Microbacterium oxydans*	Actinobacteria	100	99.86	1389	MN128753
Activated Sludge	Static	EBS 1	MM with Bezafibrate	*Acinetobacter* sp.	Proteobacteria	100	99.56	1360	MN128734
		EBS 2	MM with Bezafibrate	*Pseudomonas* sp.	Proteobacteria	99	98.85	1398	MN128767
		EBS 4	PCA	*Acinetobacter* sp.	Proteobacteria	100	99.78	1354	MN128735

Activated Sludge	Static	EBS 5	PCA	*Pseudomonas* sp.	Proteobacteria	100	99.60	990	MN128768
		EBS 6*	PCA	*Pseudomonas* sp.	Proteobacteria	99	98.64	1396	MN128769
		EBS 7	PCA	*Hydrogenophaga* sp.	Proteobacteria	100	99.78	1387	MN128748
		EBS 8	PCA	*Acinetobacter* sp.	Proteobacteria	100	99.28	1381	MN128736
		EBS 9*	PCA	*Pseudomonas* sp.	Proteobacteria	100	98.68	1359	MN128770
	Agitation	EBO 1	MM with Bezafibrate	*Pseudomonas* sp.	Proteobacteria	100	99.80	988	MN128765
		EBO 2	MM with Bezafibrate	*Brevundimonas* sp.	Proteobacteria	100	100	911	MN128743
		EBO 3	MM with Bezafibrate	*Achromobacter* sp.	Proteobacteria	100	99.57	1401	MN128730
		EBO 4	PCA	*Pseudomonas* sp.	Proteobacteria	100	99.43	1402	MN128757
		EBO 5	PCA	*Brevundimonas* sp.	Proteobacteria	100	100	911	MN128744
		EBO 6	PCA	*Pseudoxanthomonas* sp.	Proteobacteria	100	99.44	1419	MN128774
		EBO 7	PCA	*Alicycliphilus denitrificans*	Proteobacteria	100	99.06	1376	MN128731
		EBO 8*	PCA	*Paracoccus* sp.	Proteobacteria	100	97.30	1327	MN128756
		EBO 9	PCA	*Bosea* sp.	Proteobacteria	100	99.56	1358	MN128741
		EBO 10	PCA	*Pseudomonas* sp.	Proteobacteria	100	99.80	990	MN128766
		EBO 11*	PCA	*Amaricoccus* sp.	Proteobacteria	99	97.52	1330	MN128738

2.4.2 Biodegradation experiments

Five consortia were assembled with the isolated bacterial strains obtained from enrichment cultures with paroxetine and bezafibrate, to evaluate their capacity for the biodegradation of these compounds, under static (4 consortia) and agitation conditions (1 consortium), along 3 feeding cycles of 2 weeks. All experiments were carried out with mineral salts medium which, due to its composition, has some buffer capacity. Our studies revealed that pH in the cultures was around 7 without significant variations.

For the consortia incubated under static conditions, different removal efficiencies were observed between the consortia of isolated strains derived from different inocula (estuarine sediment or activated sludge) (Figure. 9).

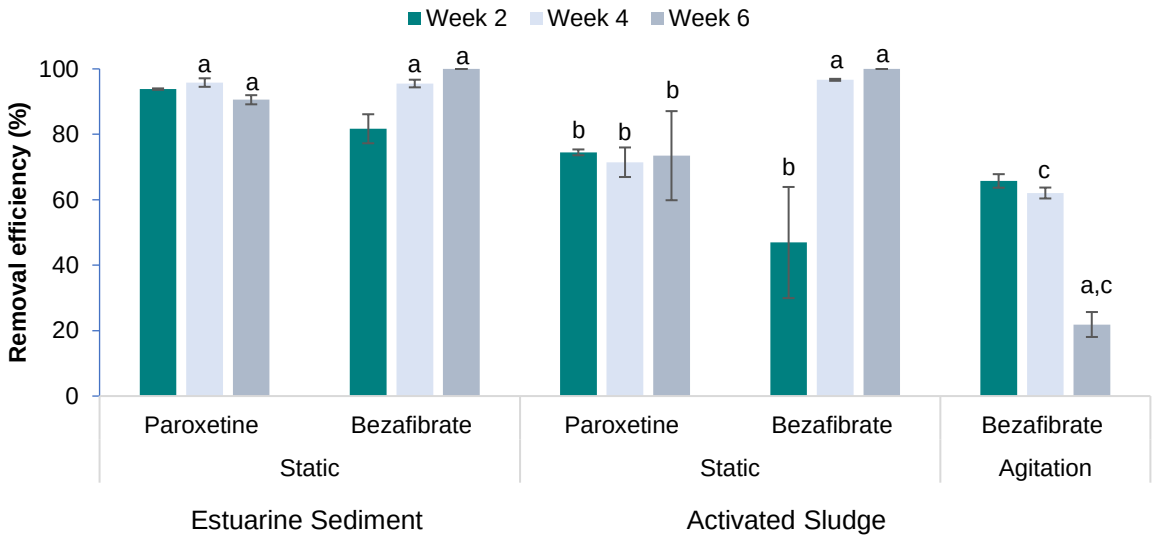

Figure 9 - Removal efficiency of paroxetine and bezafibrate by the consortia derived from estuarine sediment and activated sludge, along three feeding cycles. Week 2 – end of 1st cycle, week 4 – end of 2nd cycle, Week 6 – end of 3rd cycle. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences among the same inoculum, at different cycles ($p<0.05$); b - significant differences within the different inocula, for the same pharmaceutical and at the same cycle ($p<0.05$); c – significant differences among the same inoculum, for different incubation conditions ($p<0.05$).

In the case of paroxetine removal, the differences were significant through the entire experiment (3 cycles), while for bezafibrate removal the differences were significant only in the 1st cycle. Results revealed that consortia derived from estuarine sediment removed, in the 1st cycle, 95% and 80% of paroxetine and bezafibrate, respectively, while consortia derived from activated sludge removed 75% and 50% of these pharmaceuticals. However, at the end of the 3rd cycle, all consortia showed a high removal efficiency of the target

compounds (above 90%), except for the consortium derived from activated sludge and doped with paroxetine, for which a removal efficiency of ca. 75% was observed (Figure 9).

In addition, biodegradation of bezafibrate was also tested under agitation conditions with a consortium derived from the culture enriched from activated sludge with bezafibrate. This mode of incubation clearly led to a much lower biodegradation performance of this compound (Figure 9). Though, comparing to static conditions, no significant differences were observed in the removal of bezafibrate at the end of the 1st feeding cycle, subsequent feedings indeed resulted in significant differences. At the end of the 3rd feeding cycle, only 22% of the bezafibrate fed to the consortium was removed under agitation conditions, which highly contrasts with the removal of >97% obtained for this compound under static incubation (Figure 9). For the case of paroxetine, biodegradation was also followed by monitoring fluoride release. Results showed that for the consortia derived from both estuarine sediment and activated sludge, defluorination increased over time, reaching at the end of the 3rd feeding cycle mean values of 100% in the consortia derived from estuarine sediment and 81% in the consortia derived from activated sludge (Figure 10).

Figure 10 - Defluorination of paroxetine by the consortia derived from estuarine sediment and activated sludge, along three feeding cycles. Week 2 – end of 1st cycle, week 4 – end of 2nd cycle, Week 6 – end of 3rd cycle. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences among the same inoculum, at different cycles ($p<0.05$); b - significant differences within the different inocula ($p<0.05$).

These results on defluorination of paroxetine reinforce the evidence that the bacterial strains isolated from the cultures enriched with paroxetine could biodegrade the target pharmaceutical when assembled in consortia. Differences in the performance can be

due to the source of inoculate. Activated sludge and estuarine sediment were chosen as inoculate, both with different bacterial dynamics and composition. Thus, this can lead to a different community enrichment and isolation (as represented in Figure 5), resulting in different bacterial consortia with different degrading potential. Other experimental factors such as acclimation needs, more specifically reorganization and adaptation of the community, and isolation process, could had influence in the optimal conformation and performance of the bacterial consortia.

2.5 Discussion

The need for new biotechnological tools to remove pharmaceuticals from the environment, with less harm and negative impacts, has emerged in the last years. The use of autochthonous microorganisms can be a sustainable solution to tackle this problem. To develop this technology, degrading microorganisms must be selected from the contaminated site. Microbial enrichments consisting in the exposure of the natural communities to the selected pharmaceutical(s), are essential to select the key degrading microorganisms.

In this study, five enriched cultures obtained in a previous work (Duarte et al., 2019), four obtained in static conditions and one in agitated conditions, were selected and their bacterial constituents were retrieved by spreading the cultures in two different solid media (PCA and MM with bezafibrate or paroxetine).

In total, we were able to isolate 48 phenotypically distinct bacterial strains from the five enriched cultures. However, this number may not reflect the total number of strains present in the enriched cultures, because only a small portion of bacteria is culturable (Vartoukian et al., 2010), and some strains may not grow properly in solid media. In fact, comparing with the data reported in Duarte et al. (Duarte et al., 2019) regarding the number of OTUs (operational taxonomic units) in each enriched culture, only a small part of the community was recovered. Yet, the recovered bacteria are the most relevant in terms of biotechnology, since the culturable bacteria can be used to produce bacterial biomass for future application for in *situ* bioremediation of contaminated sites. Moreover, even though these bacterial strains showed different phenotypic characteristics, they can be the same microorganism since in different media they can present different phenotypic traits. As an example, the strain DPS2 isolated in MM media with paroxetine and the strain DPS 7 isolated in PCA (Table 1) presented 99.9% of similarity among them. The same occurred among the strains DBS 6 and DBS 7 (99.9%), EBS 1 and EBS 8 (99.9%), EBO 1 and EBO 10 (100%), and EBO 2 and EBO 5 (100%) (Table 2).

The type of pharmaceutical compound used for the enrichments (paroxetine or bezafibrate) affected the selection of the microbial community, with different bacterial strains being selected according to the drug to which they were exposed. Proteobacteria was the dominant phylum followed by the phylum Bacteroidetes. In addition, the phylum Actinobacteria was also found in the enriched cultures from estuarine sediment. Proteobacteria is a very diverse phylum and has been described as the most representative phylum in both marine and soil environments (von Mering et al., 2007). This phylum is commonly dominant in biological wastewater treatments, due to the key role of many of its representatives in organic and nutrient removal (Cydzik-Kwiatkowska & Zielińska, 2016; Vasiliadou et al., 2018). It is also well known that the phylum Proteobacteria is one of the most relevant phyla involved in the biodegradation of different pollutants (e.g., hydrocarbons (Shahi et al., 2016) and pharmaceuticals (Alexandrino et al., 2017; Vasiliadou et al., 2018)). Different incubation conditions, static and agitation, could also have influenced the enrichment process and selection. The use of different incubation conditions allows the establishment of different biochemical environments, leading to the enrichment of different degrading bacteria. In agitated conditions, the oxygen is gradually dissolved throughout the medium. On the other hand, in static conditions, as oxygen is not homogeneously dissolved, an oxygen gradient is created allowing the growth of bacteria with different oxygen requirements. So, incubation conditions, not normally addressed in current studies, can have a significant impact on bacteria selection and on the biodegradation outcome.

Looking at the genus level, the genus *Pseudomonas* was the most dominant, followed by *Acinetobacter*. Both genera have been often associated to the biodegradation of different pharmaceuticals. Degradation of acetaminophen, paracetamol, cefalexin, sulfamethoxazole, by the genus *Pseudomonas* has been reported by several authors (Gusseme et al., 2011; Jiang et al., 2014; Lin et al., 2015; Zhang et al., 2013). Wang et al. reported complete degradation of sulfamethoxazole, sulfadiazine and sulfamethazine by an *Acinetobacter* sp. isolated from activated sludge, which was acclimated with sulfamethoxazole for 3 months (Wang et al., 2018). In another study, an *Acinetobacter* strain isolated from seawater proved to be efficient in the degradation of sulfonamides (Zhang et al., 2012), showing that this genus has a high ability to degrade sulfonamide antibiotics in different environmental sites. Regarding the other genera retrieved from the cultures enriched with paroxetine and bezafibrate, these have also been associated to the degradation of different pharmaceuticals. A strain of the genus *Achromobacter*, isolated from a wastewater, was reported to degrade sulfamethoxazole and other sulfonamides (Reis et al., 2014). A *Microbacterium* strain, isolated from a soil sample, was also reported

to degrade sulfamethoxazole (Topp et al., 2013). Woźniak-Karczewska et al. reported two bacterial strains belonging to the genus *Ochrobactrum* (isolated from a soil sample and an activated sludge enrichment) capable of successfully degrading piracetam (Woźniak-Karczewska et al., 2018). *Pseudoxanthomonas* and *Leucobacter* species obtained from a biofilm developed on granular activated carbon obtained from an activated carbon filter system treating antibiotic-polluted lake water were found to be associated with the dissipation of ciprofloxacin (Liao et al., 2016). Strains of the genera *Paracoccus* and *Bosea,* isolated from activated sludge, were also identified in studies involving the degradation of the pharmaceutical's ibuprofen and ketoprofen, albeit both strains presented low degradation capacity (around 15% after 100h of incubation) (Almeida et al., 2013). Mao et al. (2018) investigated the degradation of sulfapyridine and sulfamethoxazole by two strains of *Shewanella* and obtained degradation values around 20% and 60% for sulfapyridine and sulfamethoxazole, respectively, after 5 days of incubation (Mao et al., 2018). Deng et al. reported the presence of strains of the genera *Hydrogenophaga* and *Leadbetterella* in a sulfadiazine-degrading consortium derived from activated sludge (Deng et al., 2018). A strain of the genus *Brevundimonas,* isolated from municipal WWTP composting, was reported to be capable of degrading azithromycin (Iranzo et al., 2018). A strain of the genus *Amaricoccus*, derived from activated sludge, was reported to have the potential to co-metabolize sulfamethoxazole (Kor-Bicakci et al., 2016). Despite all these studies, to our best knowledge, none of these strains have been associated before with the degradation of the two pharmaceutical compounds selected for this study (paroxetine and bezafibrate). Moreover, for some of the isolated bacterial strains, no previous association to pharmaceuticals degradation has been found. However, some of these strains have been associated with the biodegradation of other organic pollutants. The relevance of *Dyadobacter* in xenobiotic biodegradation is not yet clear however, Willumsen et al. found two strains directly isolated from soils (with no enrichment), that could have a role in the degradation of nitrogen-containing heterocyclic aromatic hydrocarbons (Willumsen et al., 2005). *Herminiimonas* species have been associated with the degradation of several haloacetic acids (monoiodoacetic, monobromoacetic, monochloroacetic and dichloroacetic acids) (Zhang et al., 2009). In our study, a strain of this genus was isolated from a microbial culture enriched with the chlorinated drug bezafibrate, indicating that *Herminiimonas* may have a role in the degradation of chlorinated compounds.

For each consortium, the enrichment process was crucial to select bacterial strains with the capacity to degrade the selected pharmaceuticals. Besides that, the enrichment process allowed the selection of potential new species in all enriched cultures (strains DPS

4, DPS 8, EPS 6, EPS 8, EPS 10, DBS 3, DBS 5, EBS 6, EBS 9, EBO 8 and EBO 11 (Tables 1 and 2; Figures. 6, 7 and 8). This is a step forward in the discovery of new potential pharmaceuticals degrading bacteria and more tests must be conducted to access their role in the degradation of paroxetine and bezafibrate.

The enrichment conditions have an important influence on the bacterial community recovered from the cultures at the end of the enrichment phase. Regarding incubation conditions, agitation promotes a greater diffusion of oxygen in the media. On the other hand, under static conditions, although oxygen is still present in the media, its concentration is lower, which can promote the growth of facultative or anoxic bacteria. In the previous work (Duarte et al., 2019), incubation conditions (static *vs.* agitation) arose as one of the main factors responsible for shaping the bacterial community structure. The results on the phylogenetic identification of the strains isolated from the cultures enriched from activate sludge with bezafibrate, under agitation and static conditions, illustrate the influence of incubation conditions on the microbial composition of the consortia. For these cultures, results revealed the selection of different bacterial genera under agitation and static conditions, with the exception of the genus *Pseudomonas* (Figure.5). In addition, agitation conditions allowed the isolation of a higher number of phylogenetically distinct bacterial strains than static conditions. This result can be related with the fact that the activated sludge inoculum was derived from a reactor with continuous agitation and input of oxygen, which promoted the selection of microbial communities acclimatized to such conditions.

The bacterial strains recovered from the five enriched cultures selected for this study were assembled in five different consortia to investigate their capacity to biodegrade the pharmaceutical compound from which they were selected. Results showed that the removal of bezafibrate by the consortia varied with time, type of inoculum and incubation conditions (Figure 9). Under static incubation, the consortium derived from estuarine sediment had a better performance at the beginning of the experiment, being capable of removing a higher percentage of bezafibrate (>80%) than the one derived from activated sludge (≈55%), at the end of the first feeding cycle. However, subsequent drug additions led to a similar performance by the two consortia and, at the end of the experiment, both consortia showed high removal efficiencies (>97%). This indicates that the selected isolates had a role in the biodegradation of this compound when assembled in consortia. As tested before (Duarte et al., 2019), bezafibrate does not undergo abiotic degradation, meaning that the removal of bezafibrate was mainly due to the presence of the bacterial consortia. When incubated with agitation, the consortium derived from activated sludge and doped with bezafibrate showed the worst results in terms of removal efficiency for this compound, reaching a removal

efficiency not higher than 65%, which decreased over time. Both consortia obtained in static conditions (one derived from estuarine sediment (DBS) and another from activated sludge (EBS)) kept the removal performance comparing with the respective enriched cultures (97% for both) whereas for the consortium obtained in agitated conditions was observed a substantial decreasing (97% to 62%). This result can suggest that some of the key bacteria may have not been successfully isolated (unculturable bacteria). In addition, equal proportions of each isolate were inoculated, for practical reasons, and this may comprise a period of reorganization and adaptation for the community to reach their optimal conformation and performance. In fact, other studies have reported variations in removal of bezafibrate in traditional activated sludge systems (CAS). Radjenovic et al. reported removal efficiencies ranging between 15 and 82% in the traditional CAS treatment (Radjenovic et al., 2007), but in another study conducted by the same authors higher removal efficiencies ranging between 50 and 100% were reported (Radjenović et al., 2009). In other study, Sui et al. reported a removal efficiency of bezafibrate of 37% in a CAS treatment (Sui et al., 2011). Thus, the results obtained in present work with the consortia derived from activated sludge are in agreement with those reported by other authors, despite the different experimental conditions used.

Concerning biodegradation of paroxetine, the consortium derived from estuarine sediment was able to remove more than 90% of this drug after the first feeding cycle (2 weeks of incubation), being this removal stable throughout the experiment (Figure 9). The consortium derived from activated sludge was also able to remove paroxetine, but its removal was slightly lower, reaching the maximum removal of paroxetine (71%) only at week 6 (Figure 9). Fluoride anion release was measured in the consortia doped with paroxetine as an indicator of biological degradation (Figure 10). At the end of the experiment, for both consortia, the results of fluoride release were in agreement with the percentage of paroxetine removed from the culture medium. This indicates that all paroxetine removed from the culture medium was transformed into non-fluorinated metabolites. The removal of the fluorine atom from a molecule, in this case paroxetine, usually causes a significant reduction in its toxicity, making the compound less recalcitrant and easier to degrade (Janssen et al., 2001). The consortium derived from estuarine sediment maintained the removal capability to degrade paroxetine, comparing with the removal efficiencies obtained for the enriched cultures (Duarte et al., 2019), indicating that the key bacterial strains involved in the degradation were isolated. On the other hand, the consortium derived from activated sludge decreased (97% to 75%) the degradation ability comparing with the respective enriched culture. As mentioned before, this decreasing in the

removal efficiency can be due to acclimation needs or some key bacteria lost during the isolation process. Removal of paroxetine has been shown to occur in wastewater treatments, with removal efficiencies ranging between 60 – 90% for activated sludge treatment (Kosma et al., 2019; Radjenovic et al., 2007). In addition, Radjenovic et al. also reported high removal efficiency of paroxetine from a wastewater (89%) using a laboratory-scale membrane bioreactor (MBR) (Radjenovic et al., 2007). However, paroxetine can suffer abiotic degradation, like photolysis (Kwon & Armbrust, 2004), which can contribute to the high removal efficiencies reported for this compound. In a previous study (Duarte et al., 2019), we observed a significant removal of paroxetine by abiotic processes, but no defluorination, demonstrating that the presence of microorganisms was essential for the dehalogenation of the molecule. Therefore, in the present work, the obtained results of fluoride release are a solid indication that paroxetine was removed by the consortia through biodegradation mechanisms.

Overall, in this work we show that the bacterial strains isolated from the enrichment cultures with paroxetine and bezafibrate, were capable of degrading the selected pharmaceuticals when assembled into consortia, indicating that these bacteria have an active role in the degradation of these compounds. High removal efficiency of pharmaceuticals (>97%) was observed for three of the five studied consortia. Lower removal efficiency was observed for two of the consortia derived from activated sludge (one pre-enriched with paroxetine in static conditions and another pre-enriched with bezafibrate in agitated conditions). In addition, taxonomic identification of these microorganisms allowed to understand for the first time the phylogenetic diversity associated with the biodegradation of paroxetine and bezafibrate, which, to our best knowledge, has not yet been reported. Further studies should be performed to investigate the individual potential of each bacterial isolate to degrade the selected pharmaceuticals and to optimize the production of bacterial biomass for future bioremediation applications.

2.6 Conclusions

The results presented in this study showed the potential of autochthonous bacteria derived from estuarine sediment and activated sludge of an associated WWTP to bioremediate the pharmaceuticals paroxetine and bezafibrate. The consortia derived from estuarine sediment had better biodegradation performances comparing to the consortia derived from activated sludge, and incubation under static conditions led to better results than agitation. The consortia pre-enriched with paroxetine and bezafibrate were dominated by microorganisms affiliated with the phylum Proteobacteria, where the genus

Pseudomonas was the most abundant. Several potentially novel bacterial species were also retrieved from these consortia. This work unveils for the first time the taxonomic diversity associated with the biodegradation of paroxetine and bezafibrate. The consortia developed in this work may represent an important contribution for new bioremediation tools to remove pharmaceuticals from contaminated environments.

Chapter 3

Potential of native bacterial strains from estuarine environments to degrade halogenated pharmaceuticals

3.1. Abstract

In the last years, removal of pharmaceuticals from the environment has become one of the main concerns worldwide. Autochthonous bioremediation, a technology using specialized degrading microorganisms to degrade pollutants, has shown to be a promising tool to remediate contaminated environments with different pollutants. For that, it is crucial to select microorganisms with metabolic capability to degrade the target pollutant.

This work aimed to optimize two degrading bacterial consortia, for the degradation of paroxetine and bezafibrate. For that, the potential of the bacterial strains to degrade the pharmaceutical, used for its isolation, was accessed in biodegradation experiments, either as single strains or combined in consortia. In addition, the versatility of each bacterial consortium to bioremediate the other pharmaceutical was also evaluated, by exposing the paroxetine degrading consortium to bezafibrate and bezafibrate degrading consortium to paroxetine.

Several bacterial isolates from the paroxetine degrading consortium showed degradation potential, in which *Pseudomonas* DPS 10, *Pseudomonas* DPS 1, *Chitinophagaceae family* DPS 4 and *Leadbetterella* DPS 8 were the best degrading strains with removal efficiencies between 81% – 99% and defluorination values between 64% – 77%. Up to 85% of paroxetine was removed from the media, in which ca. 80% of the molecule was defluorinated. For the bezafibrate degrading consortium, the most promising isolates (*Microbacterium oxydans* DBS 10, *Dyadobacter* DBS 3, *Ochrobactrum rhizosphaerae* DBS 4 and *Leucobacter* DBS 5) were able to remove up to 78% of bezafibrate, while the bacterial consortium displayed removal efficiency up to 67%, displaying a significant decreasing comparing with data from a previous study. Moreover, paroxetine degrading consortium was able to degrade up to 92% of bezafibrate, and bezafibrate degrading consortium displayed removal efficiency of ca. 85% for paroxetine, in which around 45% of the molecule was defluorinated. Cryopreservation and reactivation of the bacterial strain may have had an influence in the removal efficiencies observed across experiments.

The results highlighted the potential of different bacterial isolates and respective bacterial consortia, retrieved from estuarine sediment, to biodegrade paroxetine and bezafibrate. This is the first time that the degradation of these pharmaceuticals by isolated strains is described. These findings should be considered in the future in the development of bioremediation formulas for the recovery of contaminated environment.

3.2. Introduction

Microorganisms are the base of world life and essential for the maintenance of different ecosystems (Saccá et al., 2017), being key players in the biogeochemical nutrient cycling, climate management and ecosystems functioning (Chen et al., 2020; Saccá et al., 2017). In addition, they are also responsible for the degradation of different organic pollutants (Caracciolo et al., 2015; Prakash et al., 2013). In fact, their ability to grow in different environments (aerobic, anoxic and anaerobic), catabolic capacity and wide diversity have brought an increasing attention on their use for the degradation of pollutants (Megharaj et al., 2011).

Taking advantage of natural processes, bioremediation technology, relying on biological degrading mechanisms, has been explored to recover contaminated environments, aiming to transform pollutants into harmless compounds (Azubuike et al., 2016). Accessibility, bioavailability and concentration of the pollutant, as well as other environmental parameters such as temperature, pH, presence of other carbon sources and nutrient availability, are key features that influences the capability of microorganisms to detoxify the pollutant (Azubuike et al., 2016; Megharaj et al., 2011). Bioremediation tools can be developed by using autochthonous or allochthonous microorganisms. The use of indigenous microorganisms isolated from the contaminated site has been considered a promising solution for the degradation of persistent pollutants (Azubuike et al., 2016; J. Li et al., 2020). The use of allochthonous microorganisms, *i.e.*, non-indigenous microorganisms, can encompass adaptation problems and competition with the autochthonous community, affecting the bioremediation performance (Azubuike et al., 2016). In addition, by adding non-indigenous microorganisms into the contaminated site, changes in the autochthonous community can occur and may cause deleterious effects on function of the receiving ecosystem.

Biodegradation of organic pollutants, such as pharmaceuticals, has been attempted using single bacterial strains (Lin et al., 2015; Wang et al., 2018; Yang et al., 2020; Zhang et al., 2013) or bacterial consortia (Duarte et al., 2019; Facey et al., 2018; Fernandes et al., 2020; Zhang et al., 2013). Depending on the type of compound and experimental design, promising results have been observed for both strategies. However, the use of different native bacteria in a consortium can be more advantageous as the presence of different bacteria can enhance cooperative or synergistic interactions among the bacterial community and play a crucial role in the degradation of pharmaceuticals (Aissaoui et al., 2017; Zhao et al., 2015). Still, the use of bacterial consortia, when prepared by assembling different bacterial strains, may encompass several optimization processes. In fact, during

the enrichment and isolation processes, bacterial strains with no direct or indirect role in the degradation of the pharmaceutical can be selected, only because they have the capability to adapt to the new conditions. Furthermore, working with bacterial consortia made by combining different bacterial strains can be difficult in terms of scale up, as the higher the number bacterial strains the higher the costs of biomass production can be and satisfying the different metabolic needs can be a challenge. Nevertheless, for the success of bioremediation, it is crucial to select microorganisms, native of the impacted site, with metabolic capability to degrade the target pollutant, and to evaluate the degradation potential of each bacterial isolate, either as single strains or combined in a consortium. Optimisation of the consortium, *e.g.*, using only a minimum number of strains, can also be considered.

The aim of this study was to optimize two degrading bacterial consortia, with bacterial strains previously isolated in chapter 2, for the degradation of paroxetine and bezafibrate (Figure 11) (Fernandes et al., 2020). For that, the potential of each bacterial strain to degrade the respective pharmaceutical was accessed in biodegradation experiments, either as a single strain or as a consortium, in the presence of a secondary carbon source. The versatility of each bacterial consortium to bioremediate the other pharmaceutical was also evaluated, by exposing the paroxetine degrading consortium to bezafibrate and bezafibrate degrading consortium to paroxetine. The bacterial strains used in this work were isolated and purified in chapter 2 (Fernandes et al., 2020), from two bacterial cultures obtained after an enrichment process in the presence of the respective pharmaceutical, paroxetine or bezafibrate, using estuarine sediment as the source of inoculum (Duarte et al., 2019). The identification of the bacterial strains in these bacterial cultures was previously performed in chapter 2 (Fernandes et al., 2020).

Biodegradation of some pharmaceuticals, using pharmaceuticals as a sole carbon source (Moreira et al., 2018; Mulla et al., 2018) or in co-metabolism with a second organic carbon source (Amorim et al., 2014; Moreira et al., 2018; Pan et al., 2017), has been performed. Co-metabolism has been suggested as a promising approach to improve the biodegradation of recalcitrant pollutants, such as halogenated compounds (Dawas-Massalha et al., 2014; Feng et al., 2019). The addition of a co-metabolic substrate can enhance the microbial growth and induce non-specific enzymes for co-metabolism of the organic pollutants, leading to its degradation (Feng et al., 2019), a feature that should be taken in consideration. Thus, in this work co-metabolism was used with addition of an appropriate secondary carbon source.

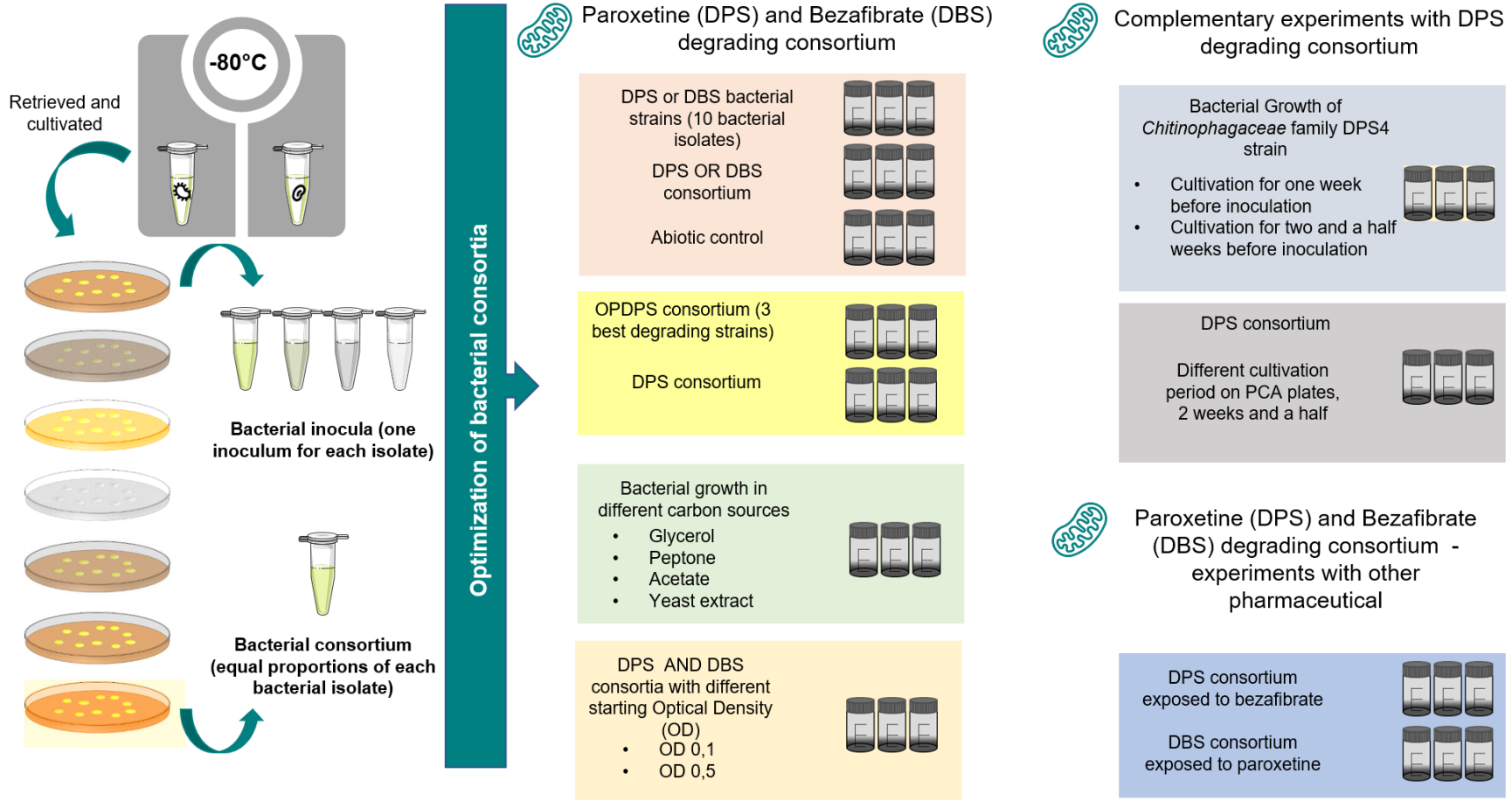

Figure 11- Schematic overview of the experiments conducted in chapter 2. OD – optical density; DPS – Paroxetine degrading consortium; DBS – Bezafibrate degrading consortium; OPDPS – optimized DPS consortium; PCA – plate count agar

3.3. Material and Methods

3.3.1. Reagents

Paroxetine HCL and bezafibrate were acquired from Enzo Life Sciences and Sigma-Aldrich, respectively. Methanol and acetonitrile HPLC grade and formic acid (98%) were acquired from Sigma-Aldrich.

To avoid biological contaminations, all material was decontaminated and/or sterilized by autoclaving the material at 120°C for 20minutes.

Compounds solutions were prepared by dissolving a known quantity of each in methanol.

3.3.2. Optimization of bacterial consortia

Previously, Duarte et. al. (2020) performed an enrichment process with paroxetine and bezafibrate, using sediment as source of inoculum (Duarte et al., 2019). Both bacterial cultures (one enriched paroxetine and other enriched with bezafibrate) have shown potential to degrade the respective pharmaceutical (Duarte et al. 2019). Then, isolation and identification of culturable bacterial strains was performed in chapter 2 (Fernandes et al., 2020). At the end, 10 bacterial isolates were retrieved from the bacterial culture enriched with paroxetine and other ten bacterial isolates were obtained from the bacterial culture enriched with bezafibrate. These strains were reassembled into a consortium to confirm that the isolated strains in combination maintained the degradation potential of the respective pharmaceuticals (chapter 2,(Fernandes et al., 2020)). All strains were afterwards kept at -80°C.

3.3.3. Optimization of a paroxetine degrading consortium (DPS)

3.3.3.1. Biodegradation experiments with single bacterial strains

To perform the biodegradation experiments, bacterial strains were retrieved from -80°C and were cultivated on plate count agar (PCA) or Mineral-salt (MM) agar, as described in Table 3. PCA and MM agar were selected taking into consideration the medium in which each bacterial strain was initially isolated from (chapter 2, (Fernandes et al., 2020)). Strains were cultivated (28°C) and kept in medium plates from one week. After that, biodegradation experiments were assembled by exposing each strain to paroxetine, in the presence of a secondary carbon source. To achieve that, each strain was inoculated, in triplicate, into 60 mL of MM medium, placed in 250 mL Schott flasks, starting with an Optical Density (OD) of 0.1 (measured by spectroscopy at λ=600nm). Afterwards, each medium was spiked with 1 mg L^{-1} of paroxetine and fed with 500 mg L^{-1} of sodium acetate. Bacterial consortium was

also assembled to compare the removal performance among the strains and the consortium in the same experimental conditions. For that, equal proportions of each bacterial strain were suspended in MM medium and was inoculated, in triplicates, in 250 mL Schott flasks containing 60 mL of MM medium, starting with an OD of ca. 0.1. In addition, to evaluate pharmaceutical abiotic removals, abiotic controls were assembled, in triplicate, containing only 60 mL of MM medium, spiked with 1 mg L^{-1} of paroxetine.

Cultures were incubated under static conditions, in the dark and at room temperature (21°C), for 4 weeks. Bacterial cultures were transferred to new sterilized flasks once a week, to avoid oxygen depletion. In addition, 500 mg L^{-1} of sodium acetate were added twice a week, to enhance metabolic activity. The removal of paroxetine, the microbial growth, pH control and fluoride ion release were assessed in 5 mL of each bacterial culture collected weekly.

Table 3 - Taxonomic identification of bacterial isolates from paroxetine degrading consortium (DPS) and bezafibrate degrading consortium (DBS), used in the biodegradation experiments, as a consortium or as a single strain. Adapted from Fernandes et al. (2020). MM - Mineral-salt agar; PCA – plate count agar; Prx – paroxetine; Bzf - Bezafibrate

Bacterial Consortium	Isolation medium	Strain	GenBank accession number
Paroxetine degrading consortium (DPS)	MM with Prx	Pseudomonas sp. DPS 1	MN128762
	MM with Prx	Bosea sp. DPS 2	MN128739
	PCA	Shewanella sp. DPS 3	MN128776
	PCA	Chitinophagaceae bacterium DPS 4	MN128745
	PCA	Acinetobacter sp. DPS 5	MN128733
	PCA	Pseudomonas sp. DPS 6	MN128764
	PCA	Bosea sp. DPS 7	MN128740
	PCA	Leadbetterella sp. DPS 8	MN128750
	PCA	Microbacterium oxydans DPS 9	MN128754
	MM with Prx	Pseudomonas sp. DPS 10	MN128763
Bezafibrate degrading consortium (DBS)	MM with Bzf	Acinetobacter sp. DBS 1	MN128732
	MM with Bzf	Herminiimonas sp. DBS 2	MN128747
	PCA	Dyadobacter sp. DBS 3	MN128746
	PCA	Ochrobactrum rhizosphaerae DBS 4	MN128755

	PCA	Leucobacter sp. DBS 5	MN128752
	PCA	Pseudomonas sp DBS 6.	MN128758
	MM with Bzf	Pseudomonas sp. DBS 7	MN128759
	MM with Bzf	Pseudomonas sp. DBS 8	MN128760
	PCA	Pseudomonas sp. DBS 9	MN128761
	PCA	Microbacterium oxydans DBS 10	MN128753

3.3.3.2. Biodegradation experiments for the optimization of DPS consortium

Bacterial strains with the best degrading potential, *Pseudomonas* DPS 10, *Leadbetterella* DPS 8 and *Chitinophagaceae* family DPS 4 (obtained from biodegradation experiments described in sub-section 3.3.3.1) were selected to compose an optimized consortium (OPDPS). Selected bacterial strains were mixed in equal proportion (OD of ca. 0.1) and inoculated in flasks containing 60 mL of MM medium, spiked with 1 mg L^{-1} or 2 mg L^{-1} of paroxetine, and fed with 500 mg L^{-1} of sodium acetate. DPS consortium was reassembled as before to compare with the performance of the OPDPS consortium in the same experimental conditions. Time of experiment, maintenance and sampling were the same as described in sub-section 3.3.3.1.

3.3.3.3. Bacterial growth with different carbon sources

To evaluate if the low degradation performance of some strains could be related with low bacterial growth, bacterial growth in sodium acetate without the pharmaceutical compound of each strain was evaluated. For that, each bacterial strain was retrieved from -80°C and were cultivated on PCA or MM for a week. Then, the isolates were inoculated in MM medium starting with an OD of ca. 0.2 and fed with 500 mg $L^{-1}$of sodium acetate. The bacterial growth was measured after 3 and 5 days by spectroscopy at λ=600nm. Cultures were fed in the day 3 with 500 mg $L^{-1}$of sodium acetate. The bacterial strains that displayed low bacterial growth in sodium acetate were tested with other carbon sources: glycerol, peptone from meet and yeast extract. For these tests, the bacterial strains were inoculated in MM medium, starting with an OD of ca. 0.1. Bacterial cultures were fed with 500 mg $L^{-1}$of the selected carbon sources. Bacterial growth was measured after 3 and 5 days by spectroscopy at λ=600nm.

3.3.3.4. Biodegradation experiments starting with different optical density

Biodegradation experiment was conducted to evaluate if the difference on OD starting point could explain the differences observed in the performance of the DPS consortium in the previous experiments (sections 3.3.3.1 and 3.3.3.2). These experiments used a lower OD than those used in chapter 2, (0.5 in Fernandes et al. (2020)), therefore rising doubts regarding the effect of the starting OD on the removal efficiency of the different experiments (Fernandes et al., 2020). So, biodegradation experiments were conducted using starting OD of 0.1 and 0.5, in which the isolates were retrieved from -80°C and cultivated in PCA or MM agar for one week. DPS consortium was inoculated in MM medium as described in 3.3.3.1. Experiment was conducted for three weeks. Maintenance and sampling were the same as described in section 3.3.3.1.

3.3.3.5. Bacterial growth with different reactivation periods for Chitinophagaceae family DPS 4

For the strain Chitinophagaceae family DPS 4, bacterial growth in sodium acetate and without the pharmaceutical compound was evaluated, in which different cultivation periods (reactivation) before inoculation were tested. For that, the bacterial strain was retrieved from -80°C and cultivated in PCA for one week (PCA 1) and for two weeks and a half (PCA 2.5) and were inoculated at the same time in MM media in the presence of sodium acetate. The bacterial growth was measured after 3 and 5 days by spectroscopy at λ=600nm. Cultures were fed in the day 3 with 500 mg L^{-1} of sodium acetate.

3.3.3.6. Biodegradation experiments for DPS consortium with different reactivation period

To verify the DPS consortium viability and if a different cultivation period (reactivation) before inoculation influences the removal efficiency, a biodegradation experiment was conducted for DPS consortium, as described in 3.3.3.1. The performance of DPS consortium was assessed by measuring the fluoride ion release. Time of experiment, maintenance and sampling were the same as described in sub-section 3.3.3.1.

3.3.4. Optimization of a bezafibrate degrading (DBS) consortium

To perform the biodegradation experiments, bacterial strains were retrieved from -80°C and were cultivated on PCA or MM agar, as described in Table 3. Strains were cultivated (28°C) and kept in medium plates from one week.

3.3.4.1. Bacterial growth with different carbon sources

Considering results from tests described in section 3.3.3.3, initially the ten bacterial strains obtained from the bacterial culture enriched with bezafibrate were grown in sodium acetate for one week, as described in section 3.3.3.3. After that, bacterial strains with low bacterial growth were grown with other carbon sources: glycerol, peptone from meet and yeast extract. The methodology is described in the section 3.3.3.3.

3.3.4.2. Biodegradation experiments with single bacterial strains

Biodegradation experiments were assembled by exposing each of the ten strains to bezafibrate, in the presence of a secondary carbon source. To achieve that, each strain was inoculated, in triplicate, into 60 mL of MM medium placed in 250 mL Schott flasks, starting with an OD of 0.1. Then, each medium was spiked with 1 mg L^{-1} of bezafibrate. The strains *Dyadobacter* DBS 3, *Leucobacter* DBS5 and *Microbacterium oxydans* DBS10 were fed with 500 mg L^{-1} of yeast extract, as optimized in the section 3.3.4.1. The other seven strains were fed with 500 mg L^{-1} of sodium acetate. Two bacterial consortia were assembled as described in section 3.3.3.1, in which one was fed with 500 mg L^{-1} of sodium acetate and the other with 500 mg L^{-1} of yeast extract. Abiotic controls were assembled with sodium acetate and with yeast extract to assess abiotic degradation of bezafibrate. Cultures were incubated under static conditions, in the dark and at room temperature (21°C), for 4 weeks. Maintenance and sampling were the same as described in sub-section 3.3.3.1.

3.3.4.3. Biodegradation experiments starting with different optical density

DBS consortium and two bacterial strains (*Microbacterium oxydans* DBS10 and *Ochrobactrum rhizosphaerae*) were selected for experiments starting with optical density of 0.1 and 0.5. Experiments were assembled as described in sections in section 3.3.3.1 and 3.3.3.4. Experiment was conducted for three weeks. Maintenance and sampling were the same as described in sub-section 3.3.3.1.

3.3.4.4. Bacterial consortia with different pharmaceuticals

The potential of each consortium to remove/degrade also the other halogenated pharmaceutical (i.e., the bezafibrate consortium potential to degrade paroxetine and vice versa) was also evaluated. The respective biodegradation experiment was assembled as described in section 3.3.3.1. DPS consortium medium was spiked with 1 mg L^{-1} of bezafibrate and DBS consortium medium with 1 mg L^{-1} of paroxetine. Both consortia were

fed with 500 mg L^{-1} of sodium acetate. Time of experiment, maintenance and sampling were the same as described in sub-section 3.3.3.1.

3.3.5. Analytical methods

3.3.5.1. Pharmaceuticals determination

Concentrations of paroxetine and bezafibrate in the bacterial cultures collected along the experiment were determined by high-performance liquid chromatography (HPLC), after a pre-treatment by solid – phase extraction (SPE) to remove the main interferants (adapted from Cavenati et al. (2012)). For that, 5 mL of each bacterial culture collected in the experiments described in sections 3.3.3 and 3.3.4 were transferred into tubes of 7 mL and centrifuge at 6500 rpm during 15 min (VWR CompactStar CS4, VWR International, USA). Afterwards, supernatant was transferred into amber glass vials and kept at -20°C until analysis. For the SPE procedure, 1 mL of supernatant was diluted in 14 mL of deionized water. Oasis HLB 30 µm, 3 cc, 60 mg Cartridges (Waters Corporation, Milford, MC, USA) were pre-conditioned with 5 mL of methanol followed by 5 mL of deionized water, using a vacuum manifold system (Supelco, Spain) coupled to a vacuum pump. Then, samples were passed through the cartridges followed by 5 mL of methanol/water mixture (5:95 v/v) and dried under vacuum conditions for 30 min. Bezafibrate and paroxetine were eluted with a solution of methanol/formic acid mixture (95:5 v/v). The eluted solution was evaporated under a gentile nitrogen stream at 30°C. Afterwards, residues were resuspended in 1.0 mL of HPLC mobile phase water/formic acid (99:1 v/v) solution.

After the SPE pretreatment procedure, samples were analyzed in a HPLC Beckam Coulter equipment (System Gold) provided with a diode array detector (module 168), an automatic sampler (module 508), and SunShell C18 2.6um 100mm X 4.6mm ID column (Chromanik Technologies, Japan). The analysis was performed with a gradient of two mobile phases, water/formic acid (99:1 v/v) and acetonitrile, both previously degassed in an ultrasonic bath. External calibration with aqueous standard solutions in mobile phase (water/formic acid (99:1 v/v)), ranging between 0.1 mg L^{-1} to 2 mg L^{-1}, were performed to quantify the amount of paroxetine and bezafibrate in solution, following a methodology previously optimized (Duarte et al., 2019).

3.3.5.2. Fluoride ion release

Defluorination of paroxetine by the bacterial consortia or by each bacterial strain was followed by measuring fluoride anion concentration in the bacterial cultures as before (Duarte et al., 2019; Fernandes et al., 2020, chapter 2). For that, bacterial culture samples collected in the experiments described in sections 3.3.3 and 3.3.4 (only experiments with

paroxetine) were transferred into tubes of 7 mL and centrifuge at 6500 rpm during 15 min (VWR CompactStar CS4, VWR International, USA). Then, 3 mL of supernatant were transferred into collection tubes and kept in -20°C until the analysis. Samples were analyzed using a fluoride ion-selective electrode (Crison 9655 C, Crison Instruments, S.A., Spain). Calibration with standard solutions containing sodium fluoride were prepared in MM medium. In addition, before the sample analysis, a total ionic strength adjustment buffer (TISAB III) was added to all samples and standard solutions in a 1:10 ratio to minimize the interferences, maintaining the ionic strength and pH constant.

3.3.6. Statistical analysis

Statistical analysis was performed using the software STATISTICA version 12 (StatSoft, Inc., 2013). In all samples, triplicates were analyzed and treated independently, and the mean values and respective standard deviations were determined. Parametric Student's t-test, using mean values and corresponding standard deviations of the replicates (n=3), was performed for both fluoride ion release and paroxetine and bezafibrate concentrations. A confidence level of 95%, *i.e.*, p-value below or equal to 0.05, was considered as a significant difference.

3.4. Results

3.4.1. Biodegradation by bacterial consortia and isolated bacterial strains

3.4.1.1. Paroxetine

3.4.1.1.1. Biodegradation experiments with single bacterial strains

Initially**,** the potential of the 10 bacterial strains, previously isolated and kept at -80°C, to biodegrade paroxetine in MM media was evaluated. Results showed that some strains displayed high removal capacity. More specifically, after 4 weeks, the strains *Pseudomonas* DPS 10, *Pseudomonas* DPS 1, *Leadbetterella* DPS 8 and Chitinophagaceae *family* DPS 4 strains displayed removal percentages higher than 80% (Figure 12). Strains *Bosea* DPS 2, *Bosea* DPS 7, *Shewanella* DPS 3, *Acinetobacter* DPS 5, *Pseudomonas* DPS 6 and *Microbacterium oxydans DPS 9,* displayed removal percentages between 68-79% (Figure 12). In general, removal percentages increased over time, particularly after the first week, although differences were not always significant ($p>0.05$).

In parallel, a DPS consortium was assembled with the 10 bacterial strains previously mentioned, being also exposed to paroxetine to compare with the efficiency of the individual strains. Results showed a removal percentage up to 87% (Figure 12), a slightly lower value

comparing with removal percentages (which were> 97%) previously observed in chapter 2 (Fernandes et al., 2020). Furthermore, only the strain *Pseudomonas* DPS 1 presented higher removal efficiency comparing with the consortium, whiles *Pseudomonas* DPS 10, *Leadbetterella* DPS 8 and *Chitinophagaceae family* DPS 4 presented slightly lower removal efficiency, even though differences were not significant ($p>0.05$). However, abiotic removal of paroxetine reached 45% after 4 weeks, showing that this pharmaceutical can be abiotically removed.

Paroxetine biodegradation was followed by measuring fluoride release, i.e., defluorination (Figure 13). Defluorination in general increased over time, although differences were not always significant. Results showed that for the paroxetine degrading consortium, almost 80% of paroxetine was defluorinated. In addition, defluorination values up to 75% were observed for strains *Pseudomonas* DPS 10, *Pseudomonas* DPS 1 and Chitinophagaceae family DPS 4 (Figure 13), showing the high potential of these strains for the degradation of paroxetine. On the other hand, the strains *Bosea* DPS 2 and *Shewanella* DPS 3 displayed the lower defluorination throughout time (<15%), although a significant increase in the fourth week for the strain *Bosea* DPS 2 (reaching ca 65%) was observed (Figure 13). For the other strains defluorination varied between 45% and 65%. But no significant defluorination percentage was observed in the abiotic controls indicating that defluorination did not occur by abiotic processes. The DPS consortium displayed the highest defluorination values, though *Pseudomonas* DPS 10, *Pseudomonas* DPS 1 and Chitinophagaceae family DPS 4 displayed similar defluorination percentages ($p>0.05$).

The pH and OD for all samples were monitored during the experiment. No significant pH fluctuations were observed during the experiment, with values around 7. Regarding bacterial growth, most strains showed a high growth throughout the experiment (Figure 14), apart from *Bosea* DPS 2 and *Shewanella* DPS 3 strains. *Shewanella* DPS 3 was the bacterial strain with lower defluorination percentage. *Bosea* DPS 2 only displayed high bacterial growth in week 4, coinciding with the significant increasing on defluorination, which suggests that cross-contamination might have occurred.

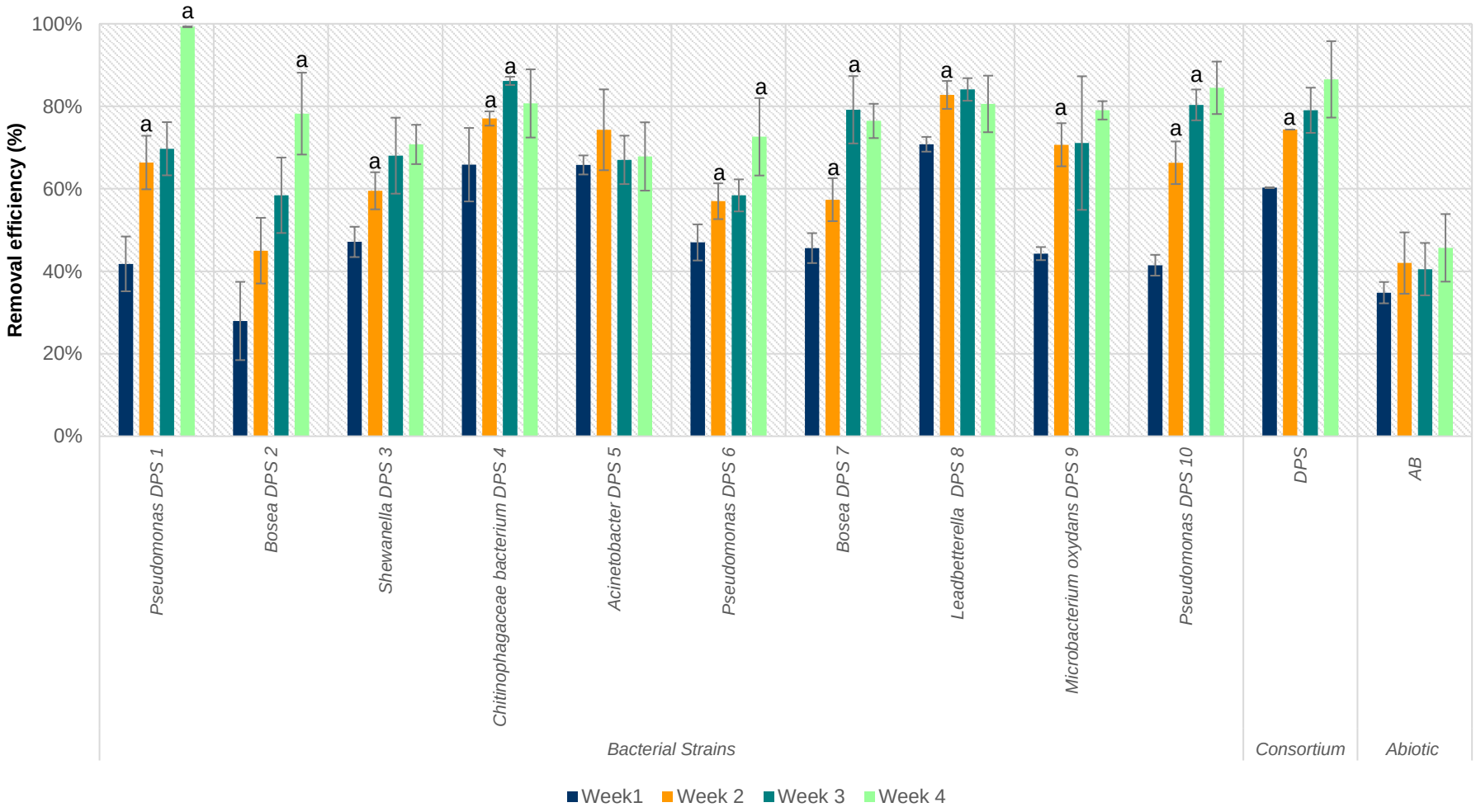

Figure 12 - Removal efficiency of paroxetine for each bacterial strain and by the paroxetine degrading consortium (DPS), after four weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a - significant differences among each treatment throughout time ($p<0.05$).

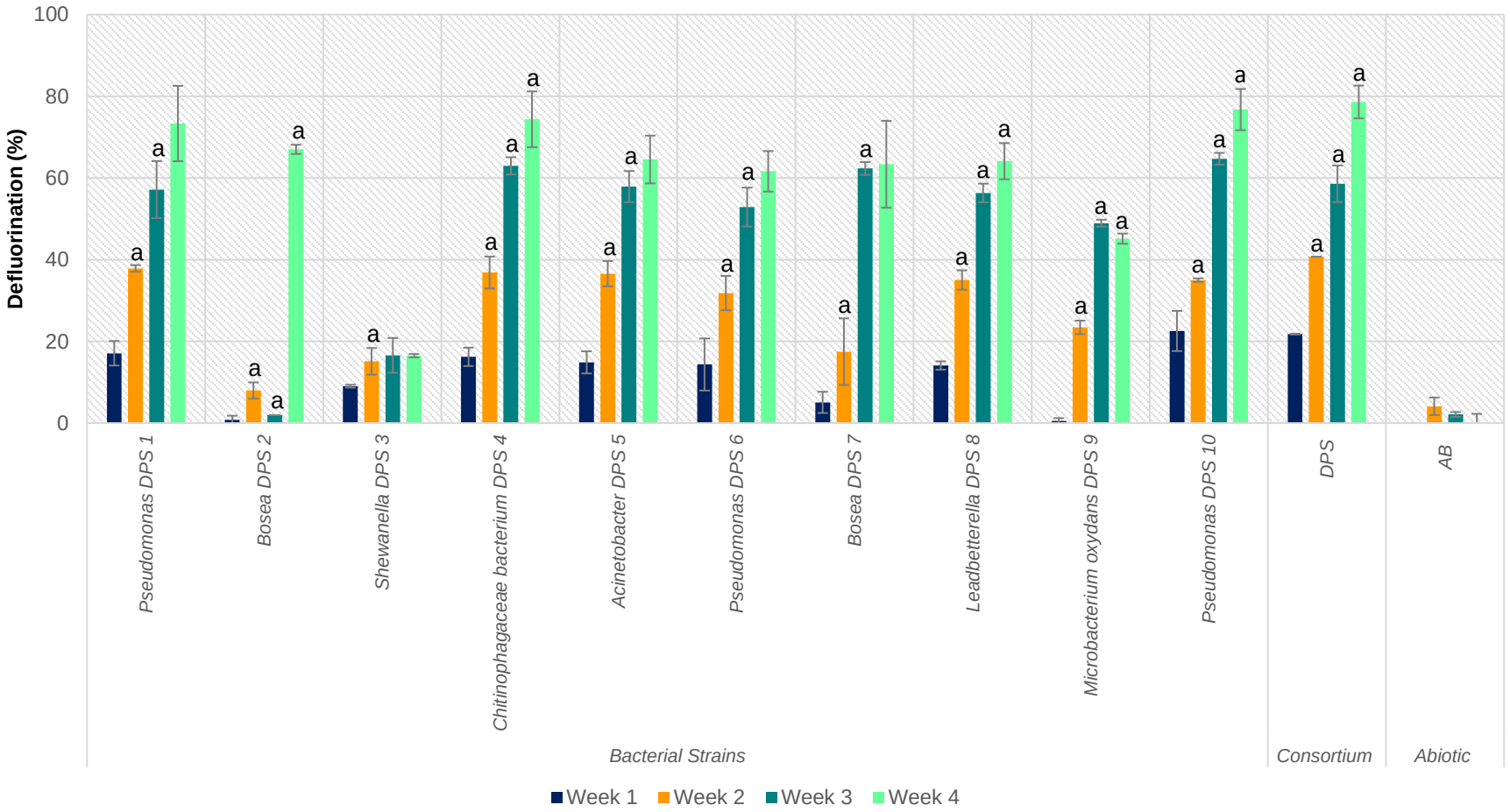

Figure 13 - Defluorination of paroxetine by each bacterial strain and by the paroxetine degrading consortium (DPS), after four weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences among each treatment throughout time ($p<0.05$).

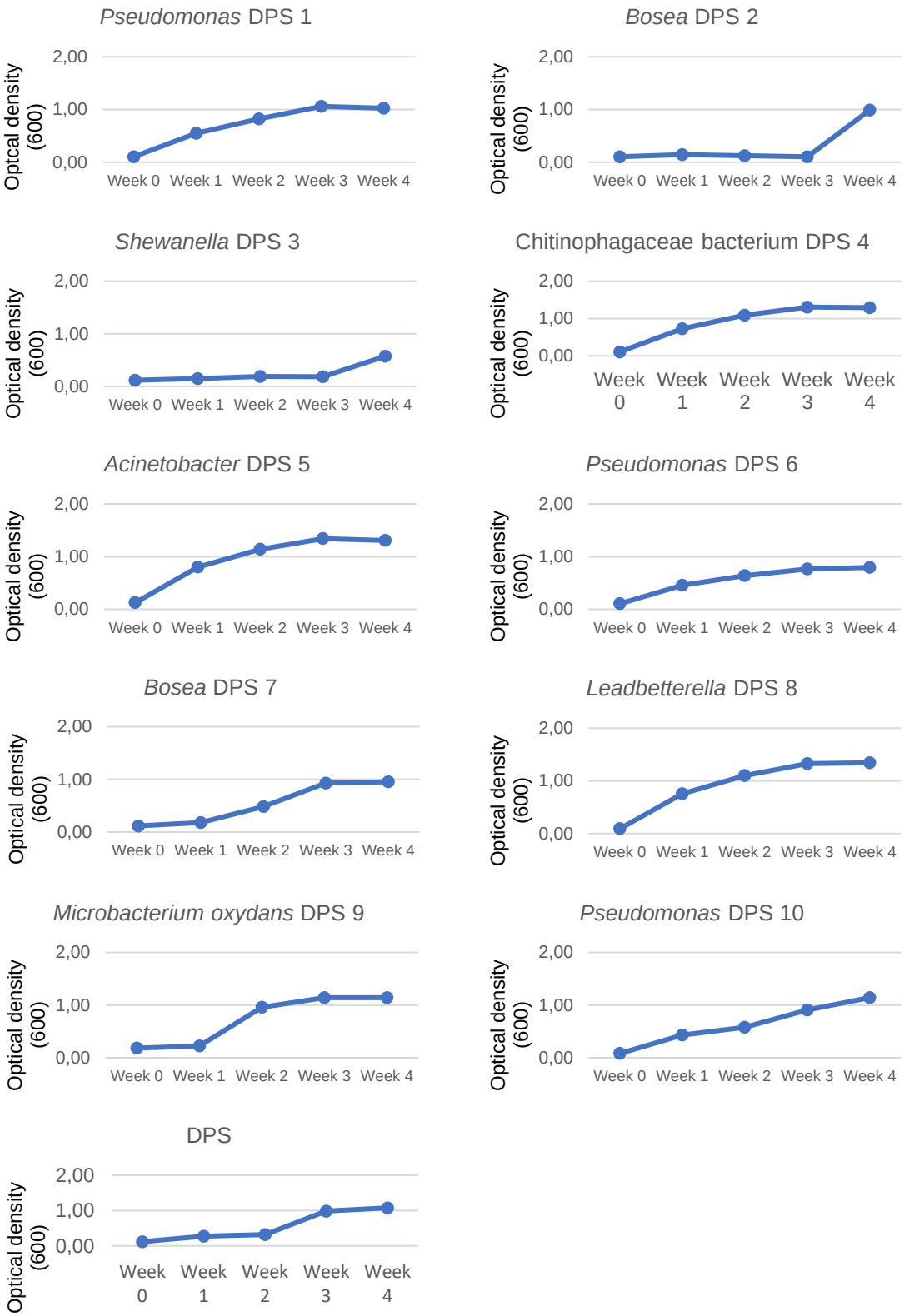

Figure 14 - Bacterial growth, determined by measurement of optical density at 600nm, of each DPS bacterial strain and DPS consortium, for 4 weeks of experiment.

3.4.1.1.2. Biodegradation experiments for the optimization of a DPS consortium

An optimized consortium (OPDPS) was assembled using the bacterial strains *Pseudomonas* DPS 10, *Leadbetterella* DPS 8 and Chitinophagaceae family DPS 4, strains that showed the higher degrading potential (Figure 12). Since *Pseudomonas* DPS 1 and *Pseudomonas* DPS 10 belong to the same genus, only one of the strains was selected to integrate the OPDPS. *Pseudomonas* DPS 10 was selected as this strain showed higher defluorination than *Pseudomonas* DPS 1 (Figure 13).

In this experiment, only defluorination was measured as an indicator of biological degradation. OPDPS consortium displayed a slightly lower defluorination than the DPS consortium (Figure 15). Nevertheless, DPS consortium showed a significant decrease on its defluorination performance comparing with the previous experience (Figure 13). Temperature and bacterial storage (-80°C) and bacterial culturing before the experiment can be related with the discrepancy among the two experiments.

Regarding the experiments performed to test the biodegradation of different paroxetine concentrations (1 mg L^{-1} and 2 mg L^{-1}) (Figure 15), half of defluorination percentage was observed for both consortia for 2 mg L^{-1} of paroxetine comparing with the respective at 1 mg L^{-1}. These results indicate that both DPS consortium and OPDPS consortium were not able to degrade a higher concentration of paroxetine.

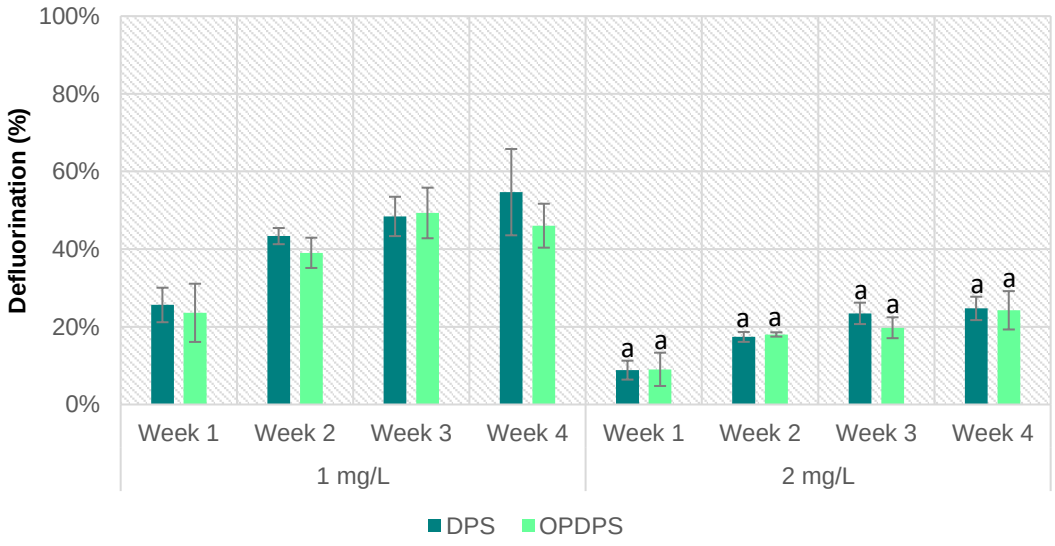

Figure 15 - Defluorination of paroxetine by paroxetine degrading consortium and optimized consortium (OPDPS), for 1 mg L^{-1} and 2 mg L^{-1}, after 4 weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences among treatment for at different concentrations ($p<0.05$).

The pH and OD for both consortia was monitored throughout the experiment. No significant pH fluctuations were observed during the experiment, with values around 7. Regarding bacterial growth, both consortia, for the concentrations, showed a high growth throughout the experiment (Figure 16).

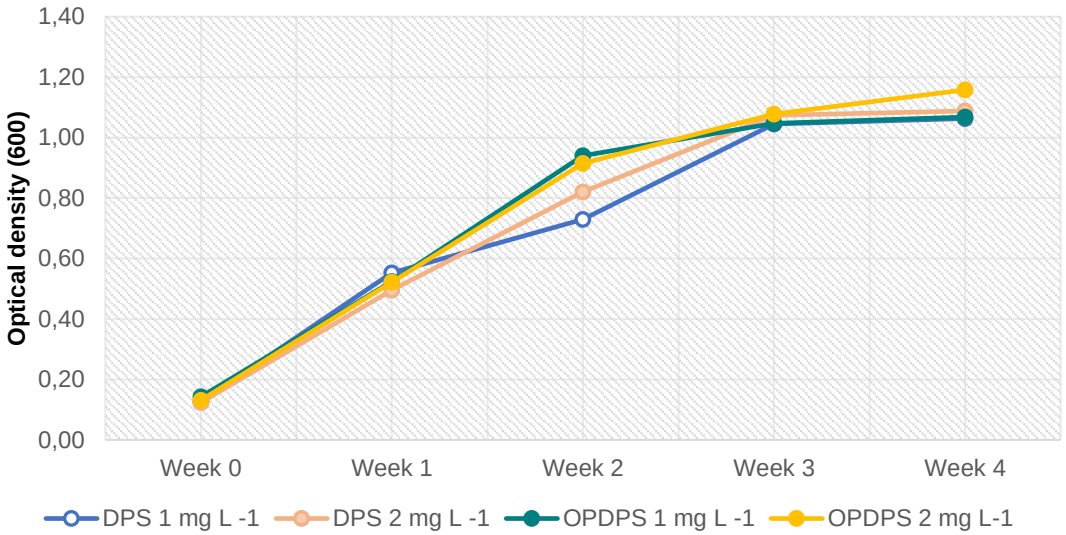

Figure 16 - Bacterial growth, determined by measurement of optical density at 600nm, for DPS and OPDPS consortia for 4 weeks of experiment.

3.4.1.1.3. Bacterial growth with different carbon sources

During biodegradation experiments with each bacterial strain, to evaluate their potential to degrade paroxetine, *Bosea* DPS 2 strain displayed low bacterial growth with sodium acetate (Figure 14). In addition, *Leadbetterella* DPS 8, Chitinophagaceae family DPS 4 and *Microbacterium oxydans* DPS 9 showed variable growth within different experiments (data not shown). To understand if these differences were related with the carbon source used as co-metabolic substrate, each bacterial strain was grown in MM media only with sodium acetate, without paroxetine. Again, *Bosea* DPS 2, *Leadbetterella* DPS 8, Chitinophagaceae family DPS 4 and *Microbacterium oxydans* DPS 9 displayed the lowest bacterial growth (Figure 17). So, for these strains, tests were carried out to evaluate their growth with other carbon sources, glycerol, peptone from meet and yeast extract, relatively to growth in sodium acetate, in the absence of the pharmaceutical compound. *Shewanella* DPS 3 also showed low bacterial growth in the experiments described in sub-section 3.3.3.1 (Figure 14), nonetheless this strain was not selected for the tests with different carbon sources as this bacterial strain was able to grow in sodium acetate in the experiments only with this carbon source (Figure 17).

Results showed that yeast extract was the most suitable carbon source for the growth of *Microbacterium oxydans* DPS 9, *Leadbetterella* DPS 8 and *Bosea* DPS 2 (Figure 18). The bacterial growth of Chitinophagaceae family DPS 4 improved in the presence of peptone and yeast extract (Figure 18).

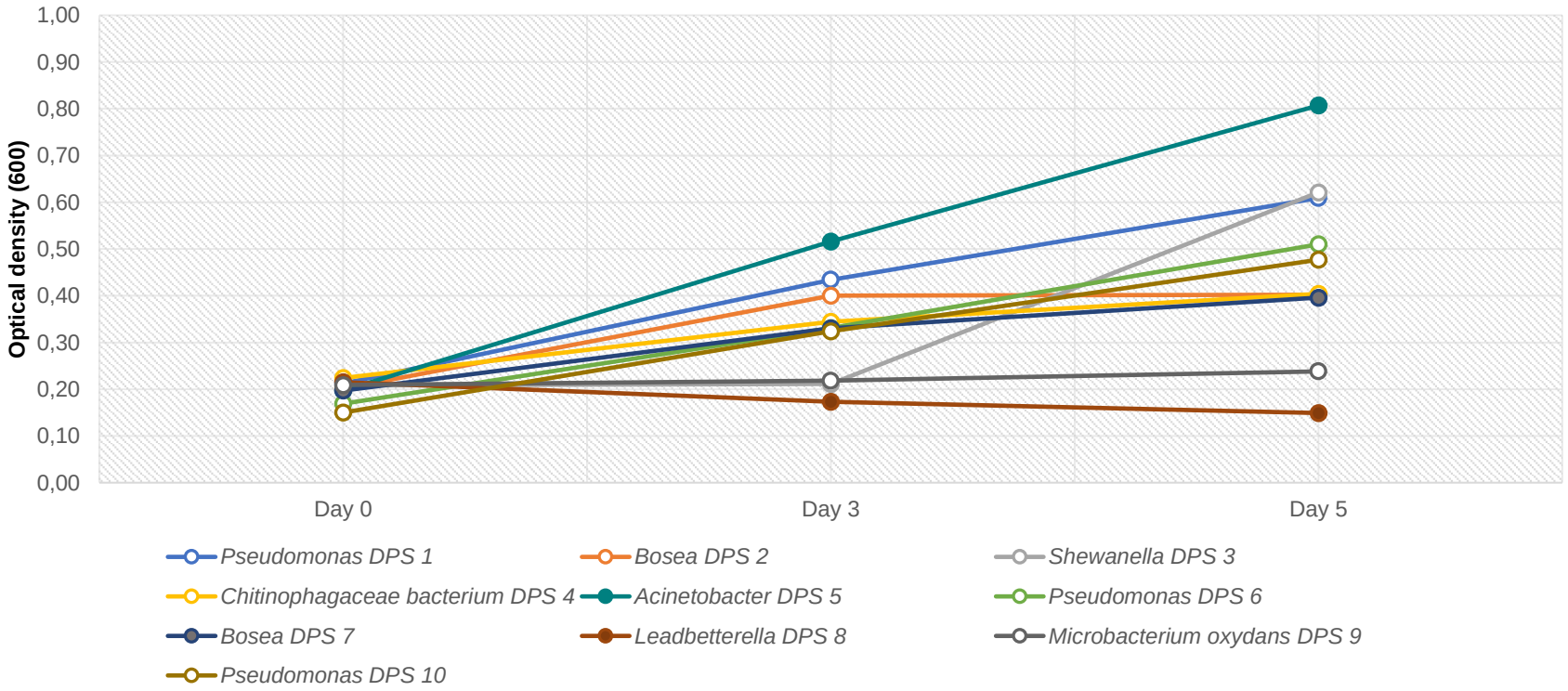

Figure 17 - Bacterial growth, determined by measurement of optical density at 600nm, for each bacterial strain of DPS consortium in Mineral-salt (MM) medium, only in the presence of sodium acetate.

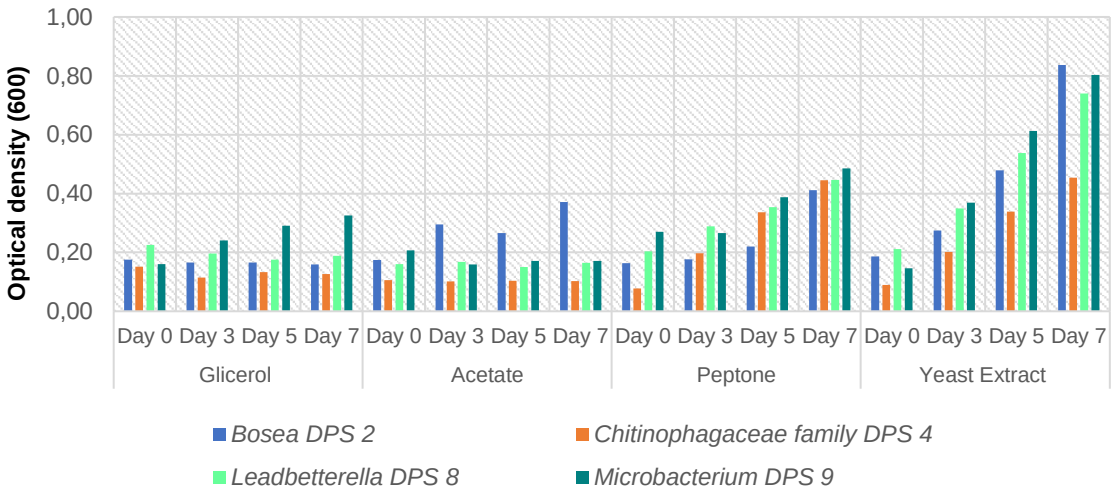

Figure 18 - Bacterial growth, determined by measurement of optical density at 600nm, for *Bosea* DPS2, Chitinophagaceae family DPS 4, *Leadbetterella* DPS 8 and *Microbacterium* DPS 9, in the presence of different carbon sources.

3.4.1.1.4. Biodegradation experiments starting with different optical density

Different optical density, 0.1 and 0.5, were tested to evaluate if the differences observed in the removal efficiency could be due to the difference of OD that was used between experiments. For both OD, only 20% of paroxetine was defluorinated by the DPS consortium (Figure 19). Compared with the previous experiments, this experiment displayed

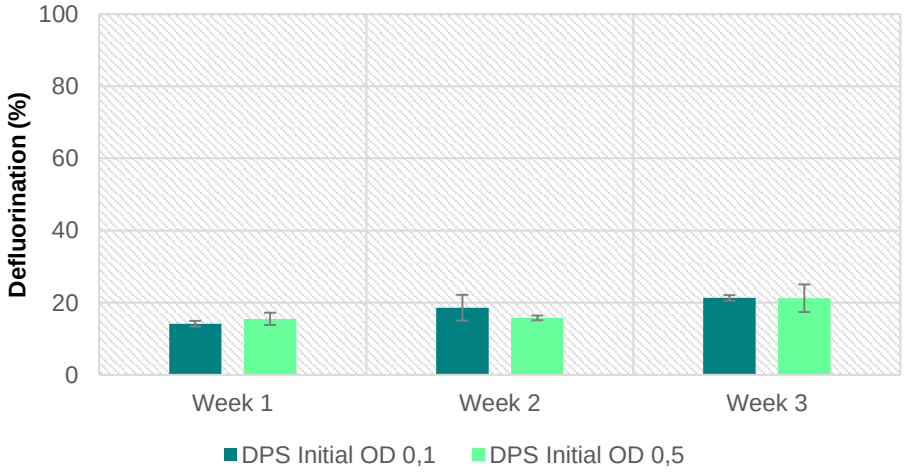

Figure 19 - Defluorination of paroxetine by paroxetine degrading consortium (DPS) starting with different optical density (OD), after three weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation.

a significant decreasing on defluorination of paroxetine, showing that cryopreservation and reactivation process can be related with the decreasing on the removal performance.

3.4.1.1.5. Bacterial growth with different reactivation periods for Chitinophagaceae family DPS 4

For different cultivation periods (1 week versus 2 weeks on a half), results showed that Chitinophagaceae family DPS 4 cultivated only for 1 week displayed half bacterial growth comparing with the Chitinophagaceae family DPS 4 cultivated for 2 weeks after 3 days (Figure 20). In day 5, both reached displayed similar bacterial growth (Figure 20) showing that time of cultivation can influenced the reactivation of the bacterial strain.

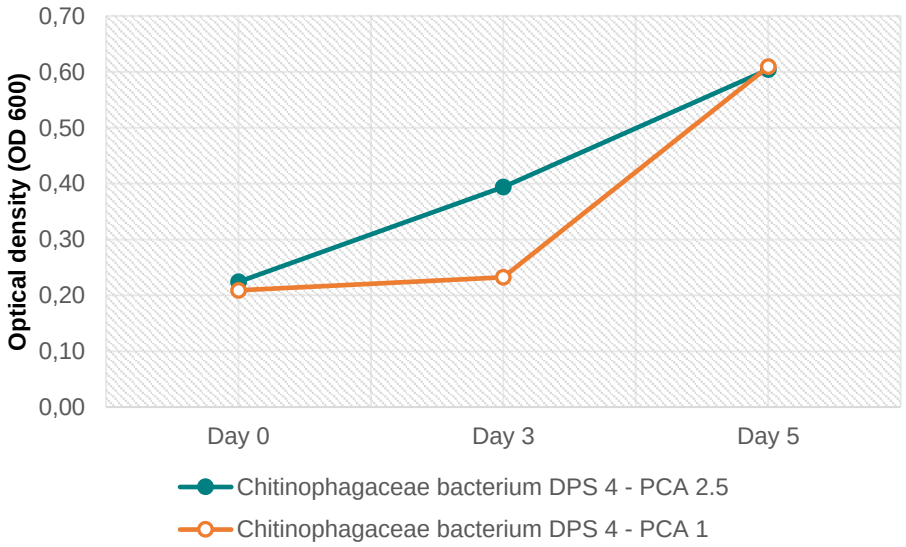

Figure 20 - Bacterial growth displayed by the Chitinophagaceae family DPS 4, for different cultivation periods, before inoculation in MM media. PCA 2.5 – Strain cultivated in PCA for 2 weeks and a half; PCA 1 – Strain cultivated in PCA for 1 week.

3.4.1.1.6. Biodegradation experiments for DPS consortium with different reactivation periods

The viability of DPS consortium was evaluated, in which the bacterial strains were retrieved from -80°C and cultivated for 2 weeks and a half. Results showed that after 4 weeks of experiment, DPS consortium was able to defluorinate 100% of paroxetine (Figure 21), reinforcing that reactivation process can influence the performance of the bacterial consortia.

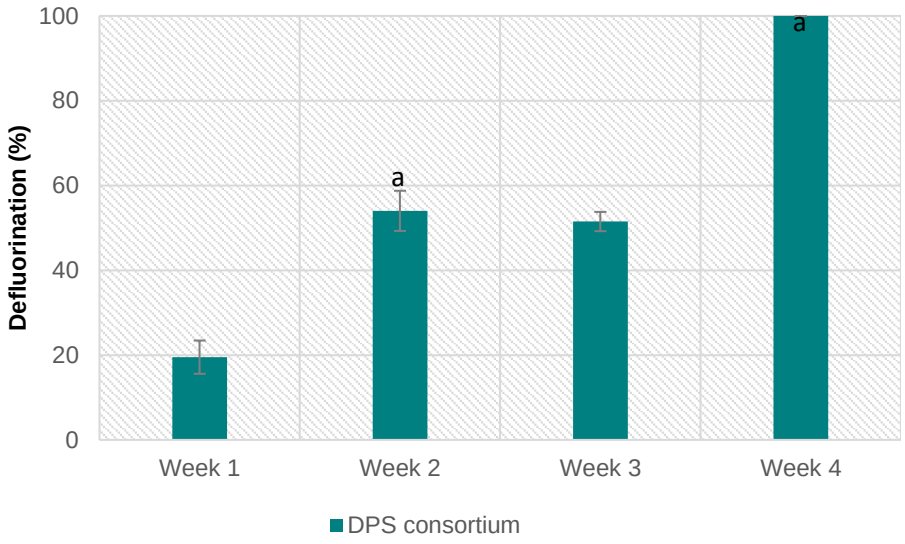

Figure 21 - Defluorination of paroxetine by paroxetine degrading consortium (DPS) after four weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences on removal efficiency throughout time ($p<0.05$).

3.4.1.2. Bezafibrate

3.4.1.2.1. Bacterial growth with different carbon sources

Before biodegradation experiments, all strains were previously grown with sodium acetate, in the absence of the pharmaceutical compound, to evaluate their ability to use it as a carbon source (Figure 22). The strains *Leucobacter* DBS 5, *Herminiimonas* DBS 2 and *Dyadobacter* DBS 3 displayed low bacterial growth in the presence of sodium acetate (Figure 22). Therefore, these strains were selected to evaluate their growth with other carbon sources: glycerol, peptone from meet and yeast extract. In this regard, *Herminiimonas* DBS 2 had the highest bacterial growth in the presence of sodium acetate and yeast extract (Figure 23). Consequently, sodium acetate was maintained as a second carbon source in biodegradation experiments for this strain. *Dyadobacter* DBS 3 and *Leucobacter* DBS 5 displayed the highest bacterial growth when these strains were supplemented with yeast extract (Figure 23). Therefore, for both strains, yeast extract was selected to be used in the following biodegradation experiments as a second carbon source.

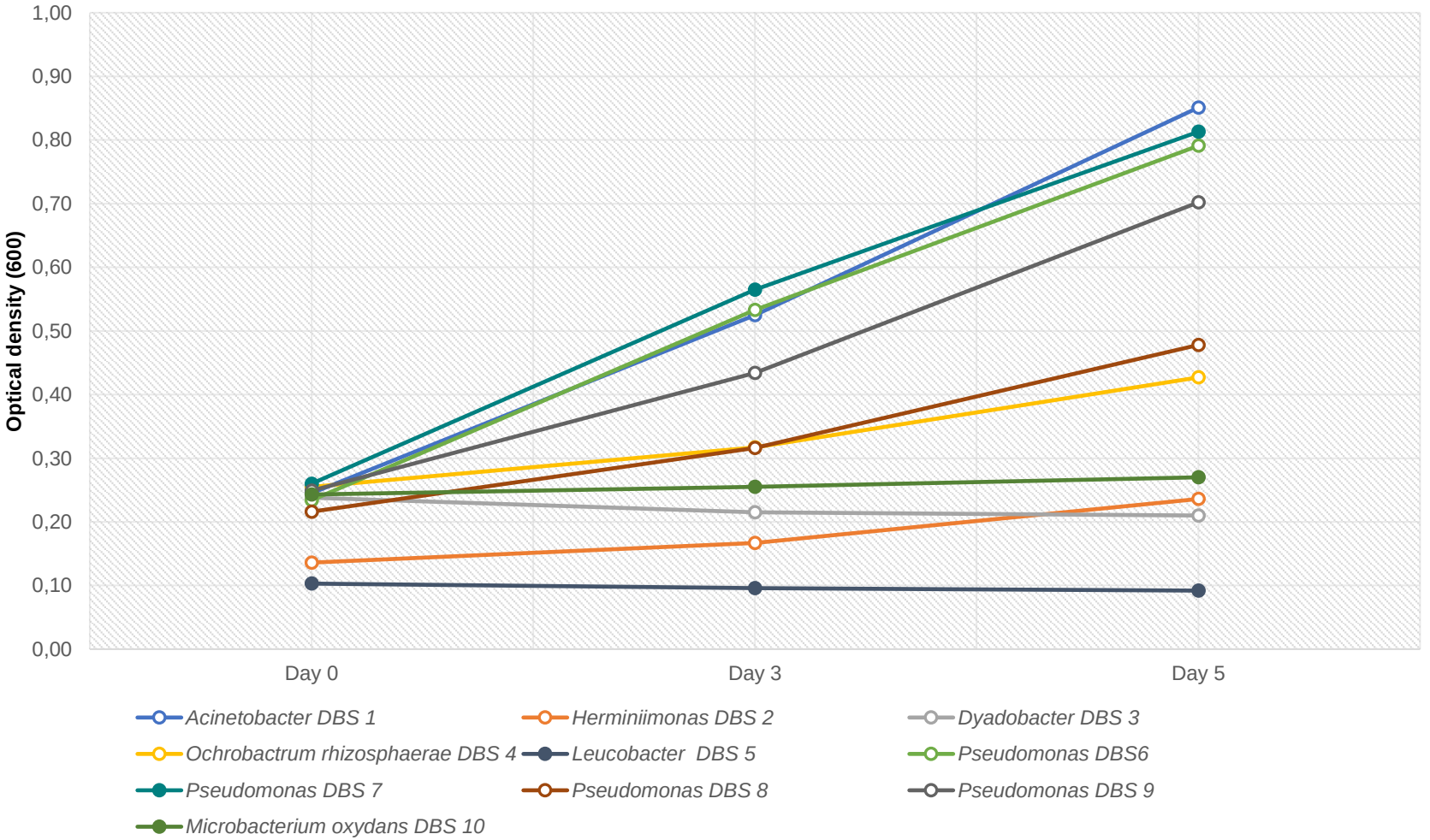

Figure 22 - Bacterial growth, determined by measurement of optical density at 600nm, for each bacterial strain of DBS consortium in Mineral-salt (MM) medium, only in the presence of sodium acetate.

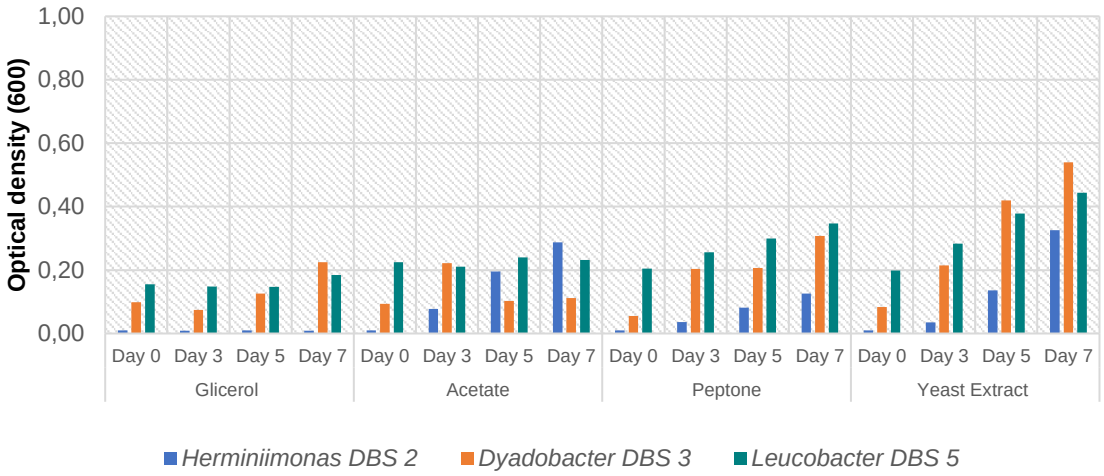

Figure 23 - Bacterial growth, determined by measurement of optical density at 600nm, for *Herminiimonas* DBS 2, *Dyadobacter* DBS 3 and *Leucobacter* DBS 5, in the presence of different carbon sources.

3.4.1.2.2. Biodegradation experiments with single bacterial strains

The potential of the 10 bacterial strains to biodegrade bezafibrate in MM media, in co-metabolisms, was assessed. Secondary carbon sources were selected accordingly to results from bacterial growth tests. All bacterial strains were fed with sodium acetate, except *Dyadobacter* DBS 3, *Leucobacter* DBS 5 and *Microbacterium oxydans* DBS 10. Yeast extract was also selected as a second carbon source for the strain *Microbacterium oxydans* DBS 10 because this strain presented 100% of similarity with *Microbacterium oxydans* DPS 9 (Table 4), a strain that presented higher bacterial growth with yeast extract than with sodium acetate (Figure 23) (section 3.4.1.2.1).

Removal efficiencies performed by the individual bacterial strains ranged between 52% and 78% after four weeks. In general, removal increased over time although differences were not always significant. *Microbacterium oxydans* DBS 10*, Dyadobacter* DBS 3, *Ochrobactrum rhizosphaerae* DBS 4 and *Leucobacter* DBS 5 presented the highest removal efficiencies, varying between 67% – 78% after four weeks of experiment (Figure 24). *Acinetobacter* DBS 1, *Herminiimonas* DBS 2 and all *Pseudomonas* strains (DBS 6, DBS 7, DBS 8 and DBS 9) displayed removal efficiencies ranging between 52% – 64% after four weeks of experiment (Figure 24).

Regarding the bacterial consortium, removal efficiency up to 67% was observed for the consortium fed with sodium acetate (Figure 24), a significant lower value comparing with the previously observed in chapter 2 (which was >97%) (Fernandes et al., 2020). Nevertheless, this removal was higher than that observed for the consortium fed with yeast.

In general, removals increased over time although differences were not always significant. No abiotic removal was observed in the presence of sodium acetate (as in a previous work (Duarte et al., 2019)) or yeast extract, indicating that biological processes were the responsible for the pharmaceutical removal. These results indicate that most bacterial strains belonging to the DBS consortium were able to degrade at least 50% of the molecule.

The pH and OD for all samples were monitored during the experiment. No significant pH fluctuations were observed during the experiment, with values around 7-8. Most of the bacterial strains presented high growth throughout the experiment (Figure 25). *Herminiimonas* DBS 2 and *Pseudomonas* DBS 8 were the bacterial strains with lower bacterial growth and *Acinetobacter* DBS1, *Dyadobacter* DBS 3 and *Microbacterium* DBS 10 were the ones with the highest growth (Figure 25).

Table 4 - Alignment scores between Paroxetine degrading strains and Bezafibrate degrading strains, among the same genera.

Paroxetine degrading strains	**Bezafibrate degrading strains**	**Pairwise Identity (%)**
Pseudomonas sp. DPS 1	*Pseudomonas* sp. DBS 6	98.3
Pseudomonas sp. DPS 1	*Pseudomonas* sp. DBS 7	98.3
Pseudomonas sp. DPS 1	*Pseudomonas* sp. DBS 8	96.6
Pseudomonas sp. DPS 1	*Pseudomonas* sp. DBS 9	96.4
Pseudomonas sp. DPS 6	*Pseudomonas* sp. DBS 6	96.9
Pseudomonas sp. DPS 6	*Pseudomonas* sp. DBS 7	96.9
Pseudomonas sp. DPS 6	*Pseudomonas* sp. DBS 8	99.1
Pseudomonas sp. DPS 6	*Pseudomonas* sp. DBS 9	95.4
Pseudomonas sp. DPS 10	*Pseudomonas* sp. DBS 6	97.4
Pseudomonas sp. DPS 6	*Pseudomonas* sp. DBS 7	97.5
Pseudomonas sp. DPS 6	*Pseudomonas* sp. DBS 8	98.8
Pseudomonas sp. DPS 6	*Pseudomonas* sp. DBS 9	95.6
Acinetobacter sp. DPS 5	*Acinetobacter* sp. DBS 1	99.5
Microbacterium Oxydans DPS 9	*Microbacterium Oxydans* DBS 10	100

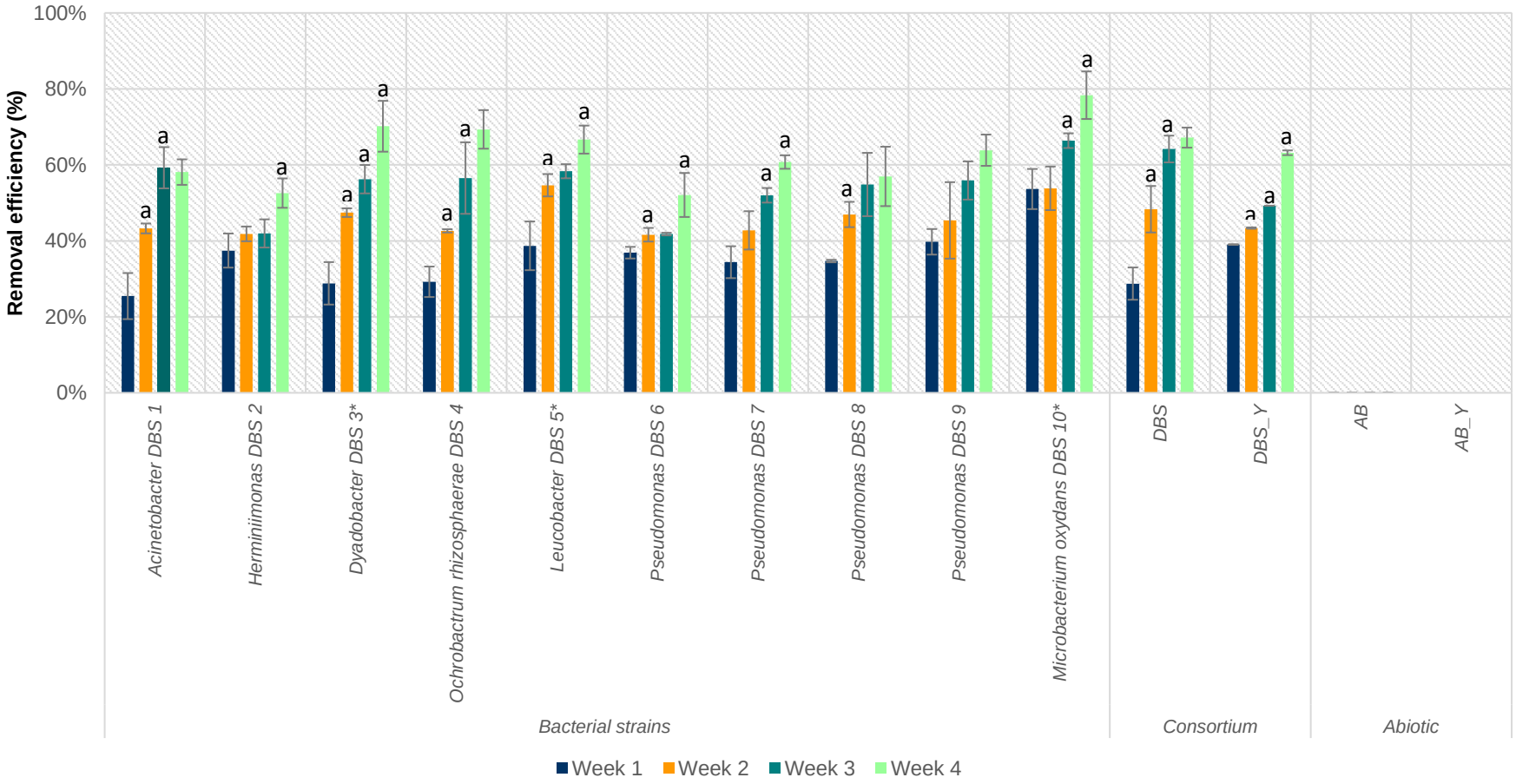

Figure 24 - Removal efficiency of bezafibrate by each bacterial strain and by the bezafibrate degrading consortium (DBS), after four weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. DBS_Y – Bezafibrate degrading consortium using Yeats extract as second carbon source. * - Bacterial isolates using Yeats extract as second carbon source. a - significant differences among each treatment throughout time ($p<0.05$).

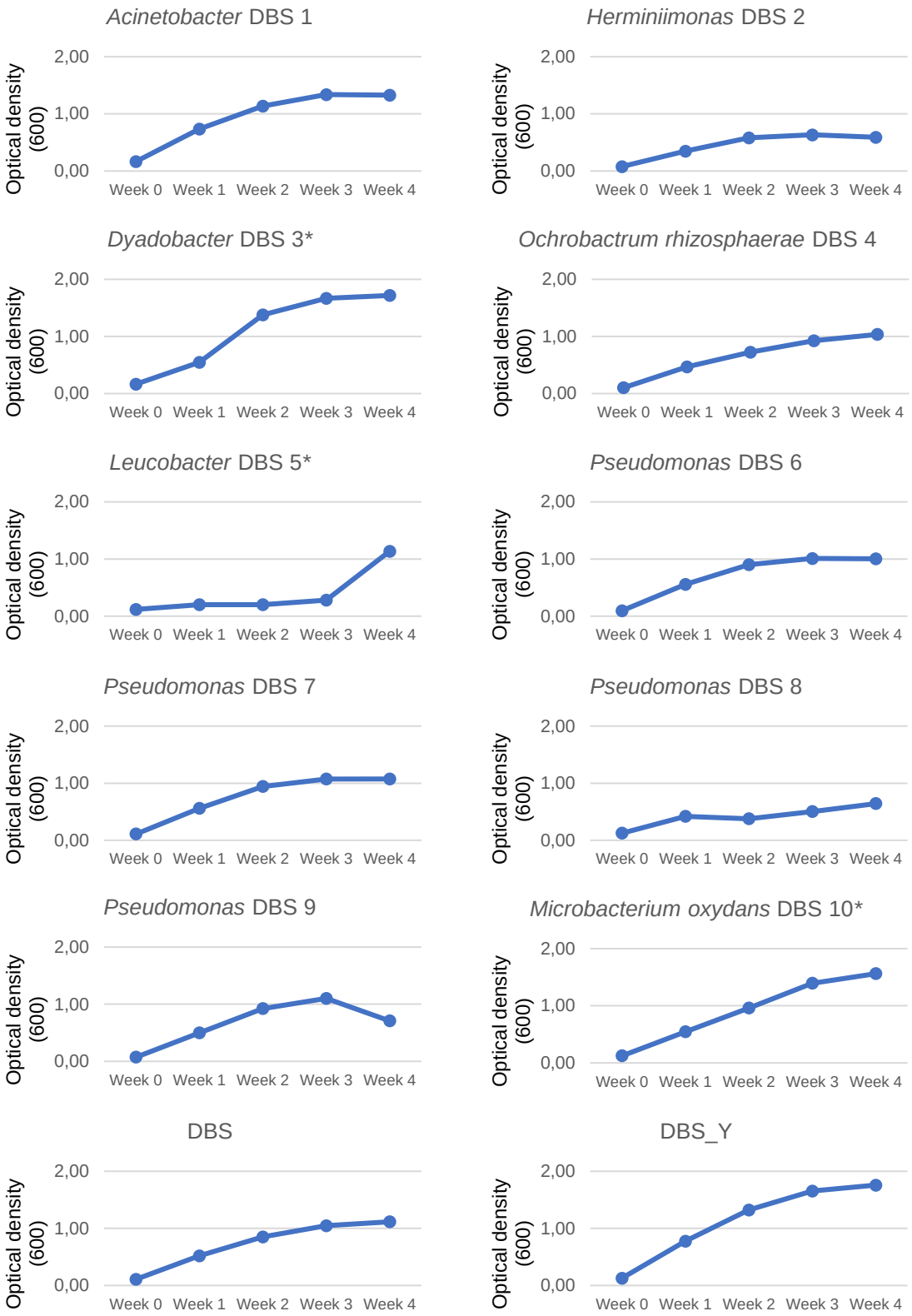

Figure 25 - Bacterial growth, determined by measurement of optical density at 600nm, of each DBS bacterial strain and DBS consortium, for 4 weeks of experiment. DBS – bezafibrate degrading consortium; DBS_Y – bezafibrate degrading consortium feed with yeast extract * - bacterial strains supplemented with yeast extract

3.4.1.2.3. Biodegradation experiments starting with different optical density

In the case of bezafibrate, DBS consortium at OD 0.1 and 0.5 displayed removals up to 38% and 45% respectively. In addition, for the two selected strains (*Microbacterium oxydans* DBS10 and *Ochrobactrum rhizosphaerae* DBS 4) removal efficiencies up to 42% and 39% were observed at OD 0.1 and up to 44% and 40% were observed for OD 0.5 (Figure S26). For both consortium and bacterial strains, a decreasing on the removal efficiency was observed. This result shows that different starting OD did not affect the removal efficiency.

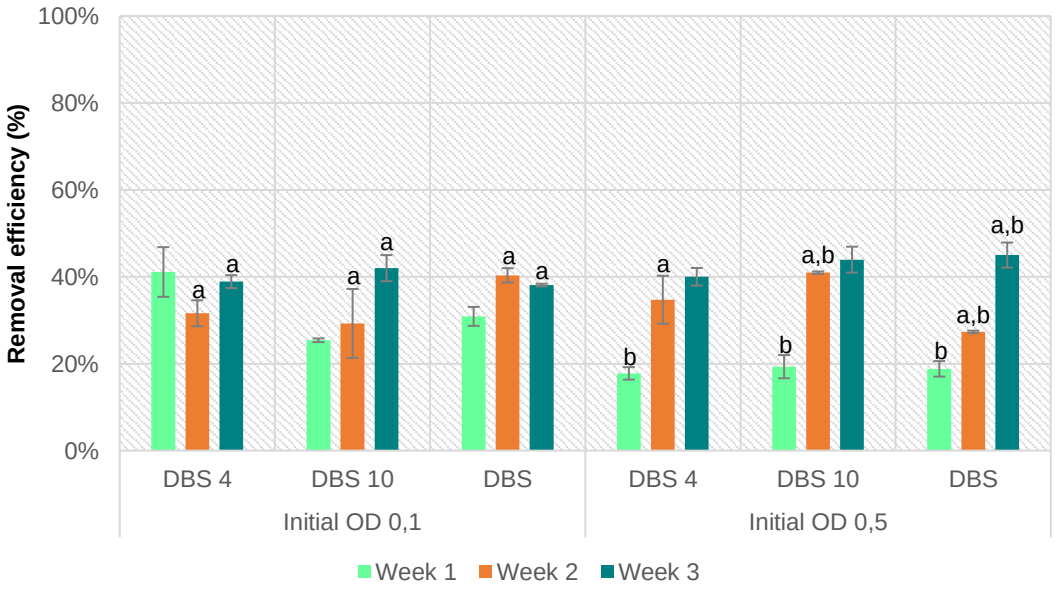

Figure 26 - Removal bezafibrate by bezafibrate degrading consortium (DBS) starting with different optical density (OD), after three weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a - significant differences among each treatment throughout time ($p<0.05$); b - significant differences in each treatment at different ODs at the same time ($p<0.05$).

3.4.2. Biodegradation experiments with other pharmaceuticals

Biodegradation of paroxetine was attempted using the DBS consortium. Results showed a removal efficiency up to 80% after four weeks (Figure 27). However, only ca. 45% was defluorinated by the DBS consortium. In general, both removal and defluorination increased with time.

Biodegradation of bezafibrate experiments using DPS consortium was also investigated. Results showed that DPS consortium displayed removal efficiency higher than 90% for bezafibrate after four weeks (Figure 28). Bezafibrate removal increased with time.

Bezafibrate did not displayed abiotic removal, indicating that the removal was performed by biological processes.

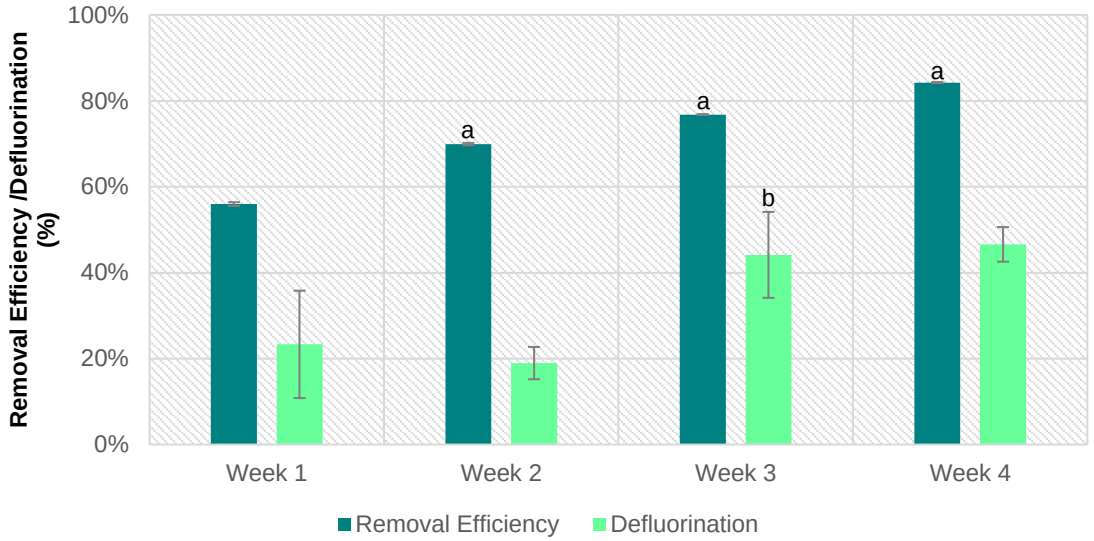

Figure 27 - Removal efficiency and defluorination of paroxetine performed by the bezafibrate degrading consortium, after 4 weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a - significant differences on removal efficiency throughout time ($p<0.05$). b - significant differences on defluorination throughout time ($p<0.05$)

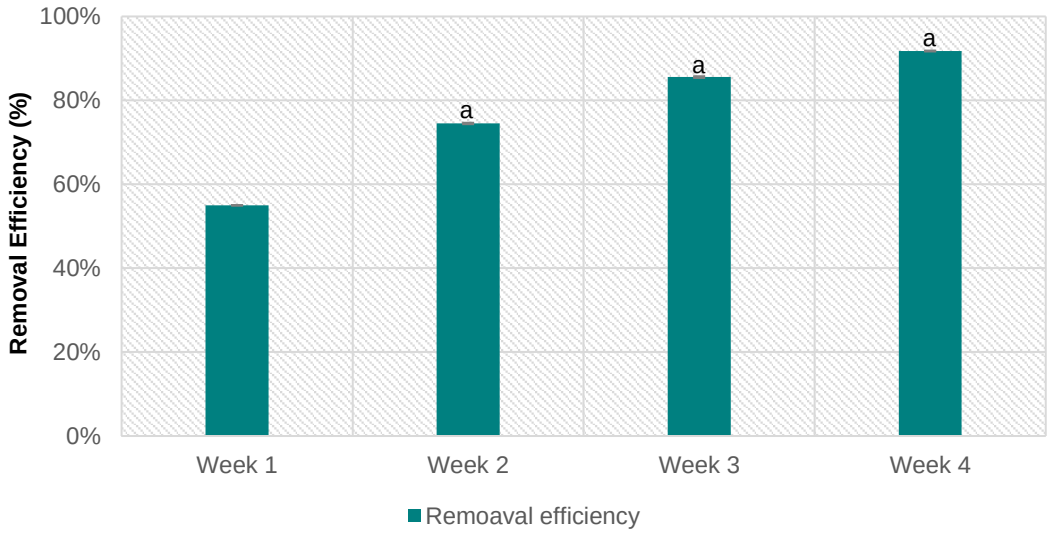

Figure 28 - Removal efficiency of bezafibrate performed by the paroxetine degrading consortium, after 4 weeks of experiment. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a - significant differences on removal efficiency throughout time ($p<0.05$).

The pH and OD for both consortia were monitored during the experiment. No significant pH fluctuations were observed during the experiment, with values around 7-8. Both consortia displayed high growth throughout the experiment (Figure 29).

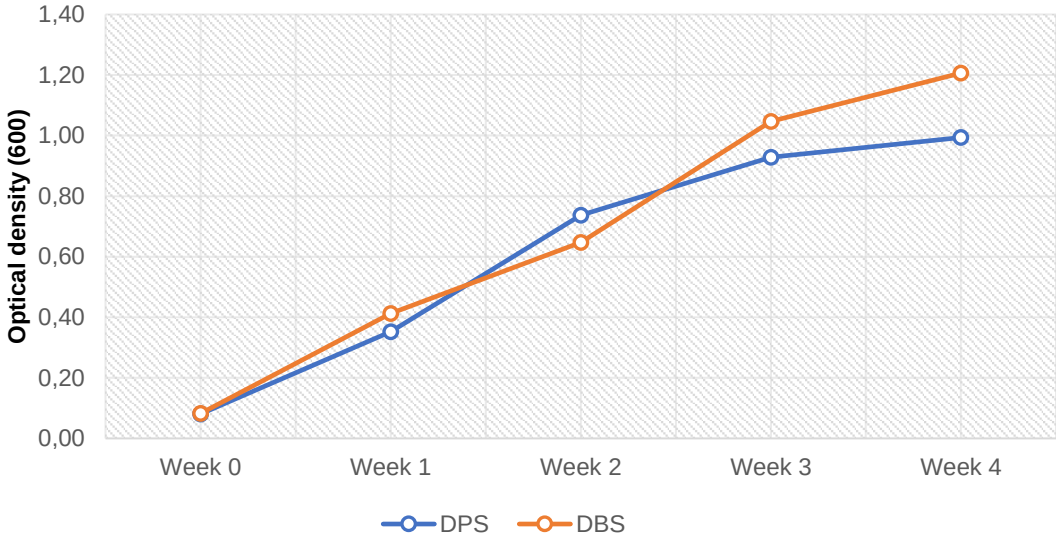

Figure 29 - Bacterial growth, determined by measurement of optical density at 600nm, for DPS (exposed to bezafibrate) and DBS (exposed to paroxetine) consortia for 4 weeks of experiment.

3.5. Discussion

In this study, the biodegradation potential of each bacterial strain isolated previously from bacterial cultures enriched in microorganisms resistant to both pharmaceuticals and stored at -80°C were assessed, aiming the optimization of a consortium with capacity to biodegrade paroxetine and a consortium with capacity to biodegrade bezafibrate. These bacterial cultures used an estuarine sediment as inoculum (Duarte et al., 2019) and showed potential to degrade the respective pharmaceutical. Isolation and taxonomic identification of the bacterial strains was performed by Fernandes and collaborators (Fernandes et al., 2020). Before storage, strains were reassembled into a consortium to confirm their potential to degrade the respective pharmaceutical.

Regarding paroxetine biodegradation experiments, in general, several bacterial strains displayed potential to degrade paroxetine. *Pseudomonas* DPS 1 and *Pseudomonas* DPS 10 showed the highest paroxetine removal (99% and 84%, respectively), followed by Chitinophagaceae family DPS 4 and *Leadbetterella* DPS 8 (removal of 81%) (Figure 12). However, defluorination was lower than removal. Moreover, paroxetine can be removed abiotically but no defluorination was observed in abiotic controls, a feature that was also reported in a previous study (Duarte et al., 2019). In addition, most strains grew over time,

in which the best degrading strains displayed high bacterial growth in the presence of paroxetine, and strains with low defluorination also showed low bacterial growth (Figure 12 and 14). Regarding DPS consortium, high removal was also observed (87%), also with lower defluorination (79%) (Figure 12 and 13). Results clearly indicate that a significant part of paroxetine removal was due to biological processes and that some strains could be more efficient for that.

Most of the bacterial strains used in the current study have been involved in biodegradation of pharmaceuticals or organic pollutants, as discusses in chapter 2 (Fernandes et al., 2020). The two strains showing higher potential belong to the *Pseudomonas* genus (strains DPS 1 and DPS 10). Biodegradation of different pharmaceuticals by the *Pseudomonas* genus have been reported by different authors (Herzog et al., 2013; Jiang et al., 2014; Lin et al., 2015; Yang et al., 2020). As an example, Yang et al. (2020) showed that two strains belonging to the *Pseudomonas* genus had capability for the degradation of the antibiotics amoxicillin, sulfamethoxazole and chlorotetracycline, in aerobic conditions (Yang et al., 2020). Other bacterial strains also showed high potential to degrade paroxetine, such Chitinophagaceae family DPS 4 and *Leadbetterella* DPS 8 (two bacterial genera belonging to the Bacteroidetes phylum) and *Acinetobacter* DPS 5 and *Pseudomonas* DPS 6 (two well-known Proteobacteria). *Acinetobacter* genus was associated with the degradation of sulfonamide pharmaceuticals (Wang et al., 2018; Zhang et al., 2012). *Leadbetterella* was found in a sulfadiazine-degrading consortium and tramadol degrading enriched culture, both derived from activated sludge inoculum (Deng et al., 2018; Kostanjevecki et al., 2019). Microorganisms belonging to Chitinophagaceae family were found in a ibuprofen-enriched community (Rutere et al., 2020). But to our best knowledge, none of these strains was reported to be involved in the degradation of paroxetine. In addition, biodegradation of paroxetine by other bacterial strains was not found in the literature.

Regarding bezafibrate, *Microbacterium oxydans* DBS 10 (78%), *Dyadobacter* DBS 3 (70%), *Ochrobactrum rhizosphaerae* DBS 4 (69%) and *Leucobacter* DBS 5 (67%) were the strains showing the higher removal percentage (Figure 24). Once again, the strains showing higher bezafibrate removal were those showing the higher growth, although removals of 40% were also observed for strains with low growth (*Herminiimonas* DBS 2 and *Pseudomonas* DBS 8) (Figure 24 and 25). For some of these strains the second carbon source (for co-metabolism) was yeast extract and not sodium acetate. DBS consortium was only able to remove up to 67% of bezafibrate, with no significant differences between the DBS consortia feed with different secondary carbon sources (sodium acetate or yeast

extract) (Figure 24). Furthermore, no abiotic removals were observed, suggesting removal was due to biological processes (Figure 24).

The bacterial strains *Microbacterium oxydans* DBS 9 and *Leucobacter* DBS 5 (two Actinobacteria), *Dyadobacter* DBS 3 (Bacteroidetes) and *Ochrobactrum rhizosphaerae* DBS 4 (Proteobacteria) displayed the best degrading performances. Biodegradation of sulfamethoxazole and sulfadiazine by *Microbacterium* sp. and *Microbacterium lacus* has been reported (Herzog et al., 2013; Tappe et al., 2013). *Ochrobactrum* sp. was reported to be involved on the degradation of erythromycin A (Zhang et al., 2017), piracetam (Woźniak-Karczewska et al., 2018) and sulfamethoxazole (Mulla et al., 2018). Biodegradation of sulfamethoxazole by a bacterial consortium composed by *Leucobacter* sp. GP and *Achromobacter denitrificans* PR1 was reported by Reis and co-authors (Reis et al., 2018). In addition, *Leucobacter* genus was found in a ciprofloxacin-degrading bacterial community (Liao et al., 2016). No data regarding the direct role of *Dyadobacter* on the degradation of pharmaceuticals was found. None of these strains were reported to be involved in the degradation of bezafibrate. Moreover, no studies involving the degradation of bezafibrate by other bacterial strains were found.

The potential of DPS and DBS consortia to degrade other pharmaceuticals was also explored. As both paroxetine and bezafibrate are halogenated compounds, the capacity of DPS consortium to degrade bezafibrate and the capacity of DBS consortium to degrade paroxetine was explored. In this regard, DPS consortium was able to remove 92% of bezafibrate from the media, showing potential to degrade other halogenated pharmaceutical (Figure 28). On the other hand, DBS consortium was able to remove 84% of paroxetine, but only 47% was defluorinated (Figure 27). Despite the lower performance comparing with DPS consortium, DBS consortium presented potential for the degradation of other halogenated pharmaceutical. Bacterial growth increased throughout time, with both consortia high bacterial growth (Figure 29). Ribeiro et al. (2012) evaluated the potential of two different bacterial consortia, one capable to degrade fluoroaromatic compound (CFB) and another from activated sludge of a municipal WWTP (CAS), to remove trimethoprim, sulfamethoxazole, ciprofloxacin carbamazepine and diclofenac (Ribeiro et al., 2012). The authors reported that none of the tested pharmaceuticals were completely removed from the media, being CAS consortium the most versatile consortium (Ribeiro et al., 2012). In addition, although high removal of ciprofloxacin (a fluorinated compound) by the CFB consortium was expected due to its ability to degrade fluoroaromatic compounds, that was not observed (Ribeiro et al., 2012).

The optimization of a DPS consortium (OPDPS), containing only the bacterial strains with the best degrading performance, was attempted. However, OPDPS consortium presented slightly lower defluorination of paroxetine comparing with the DPS consortium that was assembled with the 10 strains (Figure 15). This was observed for two different paroxetine concentrations. Results indicate that eventually all 10 strains are necessary for the degradation of the pharmaceutical despite the low performance of some strains, or that some strains that were not included in the OPDPS consortium need to be added, and for that some more studies are necessary. Both consortia, for the two tested concentrations displayed a high bacterial growth, with an increasing tendency during the time of experiment, showing that despite the lack of efficiency for the removal of paroxetine, the consortia were able to grow (Figure 16). In addition, both consortia doped with 2 mg L^{-1} were able to grow in the presence of a high concentration of paroxetine, indicating that the concentration was not toxic for the bacterial consortia despite the low removal displayed by both consortia (Figure 16). Defluorination percentage of DPS consortium in this experiment significantly decreased comparing with previous experiments. Bacterial strains used to build the DPS and OPDPS consortium had the same cultivation and inoculation conditions. Cryopreservation of the strains, their activation and adaptation after freezing may had an impact on the bacterial strains, affecting their performances overtime.

For both DPS and DBS consortia, a decreasing on the removal performance was observed comparing with a previous study, in chapter 2 (Fernandes et al. 2020). For paroxetine, a consortium assembled with the ten strains displayed removal efficiency higher than 97%, presenting complete defluorination of the molecule for the same experimental time (Fernandes et al., 2020, chapter 2). Moreover, in the present study a significant decrease on defluorination capacity was observed among different experiments. The same pattern was observed for the DBS consortium, with this consortium displaying a lower removal efficiency than that reported in chapter 2 (Fernandes et al., 2020) (removal higher than 97%). However, the consortia of Fernandes et al (2020) were assembled with the bacterial strains (10 of DPS consortium and 10 of DBS consortium) before their preservation at -80°C, which can explain the differences observed in this study.

In chapter 2, an OD of 0.5 was used to inoculate the DPS and DBS bacterial consortia. Since in the present study an OD of 0.1 was used, and a decreasing on the removal efficiency for both DPS and DBS consortia was observed, additional biodegradation experiments were assembled to test the effect of the initial OD (0.1 and 0.5) on the removal efficiency of both consortia. For bezafibrate, two selected strains were also assembled, *Microbacterium oxydans* DBS10 and *Ochrobactrum rhizosphaerae* DBS 4.

Overall, no significate differences were observed between removal efficiency of consortia or bacterial strains tested with different initial ODs (Figures 19 and 26). In addition, these experiments confirmed the decrease of removal efficiency when comparing with previous experiments. In fact, both DPS and DBS consortia showed a significant decreasing on the removal at OD 0.5, comparing with the removal obtained before with the same OD (Fernandes et al., 2020) (chapter 2). In addition, for OD 0.1, DBS consortium and two selected strains, displayed a decrease on the bezafibrate removal efficiency comparing with the removal obtained in section 3.3.4.2, and DPS consortium displayed a decrease on the paroxetine defluorination when compared with results from section 3.3.3.1. These results reinforce the hypothesis that cryopreservation and reactivation processes can affect the removal efficiency.

The lower removal percentages observed in the present study relatively to those reported in chapter 2 (Fernandes et al., 2020), could be related with the storage, reactivation of the bacterial strains or with the assemblage of the bacterial consortium. In the present study, bacterial strains were retrieved from -80°C and reactivated by culturing them in PCA or MM media accordingly to the conditions in which the strains were isolated. In chapter 2 (Fernandes et al., 2020), the bacterial strains were directly retrieved and isolated from the enriched bacterial cultures and were reassembled into the respective bacterial consortium. During the enrichment process, bacterial strains were under a selective pressure imposed by the pharmaceutical leading to the development or activation of degrading mechanisms to enable them to survive and adapt to the new environment. However, when they are cryopreserved and then reactivated, this selective pressure is not present and eventually bacterial strains could require more time to adapt and degrade the pollutant. On the other hand, by assembling the consortium with equal proportions of each bacterial isolate, the bacterial consortium can eventually require more time to adapt and reorganize, to attain the optimal composition, in terms of relative abundance of each strain, for the degradation of the pollutant.

The decreasing on the biodegradation potential can also be related with the cryopreservation of the bacterial strains. Cryopreservation of bacterial cultures has been used for the storage of cells, in which the aim is to avoid the death of cells as well as changes in their biochemical, morphological and genetic properties (Liao & Liu, 2016; Prakash et al., 2013). Cryopreservation and lyophilization (Freeze-Drying) has been widely used for the preservation of culture collections (Prakash et al., 2013). During the preservation, the bacterial strains can be subjected to severe stress and selective conditions (Lang & Malik, 1996). Throughout the process, the cells are subjected to very

low to cryogenic temperatures, promoting formation of intracellular ice, which can induce biophysical and biochemical changes and cause cryo-injuries and cell death (Day & Stacey, 2008; Prakash et al., 2013). To avoid that, the addition of cryoprotectants has been used, such as Glycerol (10–15%) and dimethyl sulfoxide (5%), among others (Hubálek, 2003; Prakash et al., 2013). However, the cryoprotects itself can pose toxicity to some organisms, a topic reviewed by Hubálek (2003) (Hubálek, 2003). Glycerol (25%) was the cryoprotectant selected for the preservation of the bacterial strains used in this study, which might not be suitable for all bacterial genera, affecting the viability and stability of the bacterial isolates. Lang & Malik (1996) evaluated the endurance and the maintenance of the biodegradation potential of six bacterial strains after preservation by freeze-drying, with and without a cryoprotectant, and by liquid-drying (Lang & Malik, 1996), reporting loss of biodegradation capability for most of the strains after the three preservation procedures (Lang & Malik, 1996).

Moreover, bacterial strains might need some time to recover from the preservation and to reactivate the degradation activity, as during preservation the metabolism of microorganism tends to diminish and can require more time to adapt to the new environment. Al-Jwaid et al. (2018) reported that two CCC samples (crosslinked cell cryogels mixed with a bacterial strain) required one week to regain their phenol-degrading activity after freezing storage (Al-Jwaid et al., 2018). In the present study, a small growth experiment with Chitinophagaceae family DPS 4 was performed. For that, bacterial strain preserved at -80°C cultivated in PCA plate for one week and bacterial strain preserved at -80°C and cultured in PCA media for 2 weeks and a half, were inoculated in MM media with sodium acetate. Bacterial strain that was retrieved from -80°C and cultivated in PCA plate only for one week required more time to growth in the MM media comparing with the one that was cultivated in PCA plate for 2 weeks and a half. In fact, after 3 days of experiment, bacterial strain cultivated for one week showed half bacterial growth comparing with one that was cultivated for 2 weeks and a half, reaching the same bacterial growth only after 5 days of experiment (Figure 20). Bacteria that was previously exposed to a specific selective pressure can display poor bacterial growth when exposed to a new environment due to a lack of certain biochemical capabilities and biophysical properties, or inappropriate expressing of those properties (Hottes et al., 2013). In addition, they can develop or lose different mechanisms, depending on the conditions to which they are exposed (Hottes et al., 2013).

To verify the DPS consortium viability, a new experiment was setup only with the DPS consortium. For this experiment, strains were retrieved from -80°C and were sub-

cultivated in the respective media for a 2 and half weeks period before being inoculated. DPS consortium was able to reach 100% of defluorination after 4 weeks of experiment (Figure 21), showing that more time for adaptation and metabolic activation of bacterial strains is needed to achieve their full performance.

Thus, all the presented factors can contribute to the variable removal degradative performances of the DBS and DPS consortia. Reactivation of bacterial strains before the biodegradation experiments should be optimized and be considered in further biodegradation studies.

This study unveiled the potential of different bacterial strains to degrade paroxetine and/ or bezafibrate, in the presence of a second carbon source. In addition, this study also demonstrated different features that can affect the removal performance of bacterial consortium, which can be a constrain for the development of bioremediation technology. Thus, future experiments should be performed addressing different constrains found in this study.

3.6. Conclusions

This work showed the potential of different bacterial strains to degrade paroxetine and bezafibrate in the presence of a secondary carbon source. For paroxetine, *Pseudomonas* DPS 10, *Pseudomonas* DPS 1 and Chitinophagaceae family DPS 4 and *Leadbetterella* DPS 8 displayed high removal and defluorination percentages, being the most promising strains for the degradation of paroxetine in synthetic media. In the case of bezafibrate, *Microbacterium oxydans* DBS 10, *Dyadobacter* DBS 3, *Ochrobactrum rhizosphaerae* DBS 4 and *Leucobacter* DBS 5 were the most promising strains, being able to degrade more than 65% of bezafibrate. DPS and DBS consortia were able to remove 87% and 67% of paroxetine and bezafibrate, respectively. A lower performance than the one observed in the previously chapter (Fernandes et al., 2020) and the results obtained in different tests indicate that a careful cryopreservation and reactivation processes are needed.

Moreover, DPS consortium showed removal efficiency higher than 90% for bezafibrate, showing the versatility of this consortium to degrade other halogenated pharmaceutical. On the other hand, DBS consortium was able to defluorinate 47% of paroxetine.

These results highlighted the potential of native bacterial strains, isolated from an estuarine sediment to degrade two different pharmaceuticals either as a consortium or as a single strain. This was the first time that the degradation of these pharmaceuticals by

isolated strains was described. These bacteria should be further considered to be used in natural media experiments (with estuarine water and sediment) to assess their ability to degrade paroxetine and bezafibrate in more a more complex media and explore their future use in bioremediation tools.

Chapter 4

Autochthonous bioaugmentation for bioremediation of paroxetine in natural media – microbial community dynamic and biodegradation potential

4.1 Abstract

Estuarine environments are very important ecosystems but also among the most sensitive and fragile. A wide range of organic pollutants can be found in these ecosystems affecting their function and the living organisms. Pharmaceuticals have been reported as pollutants of estuarine environment, thus technologies suitable to be applied in natural ecosystems are needed. Bioremediation, through bioaugmentation, can be presented as a suitable and sustainable solution to be developed and applied. This technology aims to enhance the biodegradation of the target compound, by selecting and introducing specific degrading microorganisms, with minimal long-term interference in the functionality of the natural communities. However, the effects of bioaugmentation process on the natural community dynamics should not be disregarded, being an important feature for the functioning of the affected site.

Thus, this work aimed to assess the effects of bioaugmentation process, using native microorganisms, on the dynamics of estuarine natural community, and to evaluate the potential of those microorganisms to degrade paroxetine in natural media. The native microorganisms were isolated from an estuarine sediment, through an enrichment process with the selected pharmaceutical. Microcosms were assembled, containing estuarine water and sediment collected from Douro estuary (NW Portugal), half with only estuarine water and other half with estuarine water and sediment. Microcosms were inoculated either with a bacterial consortium (composed of 10 different bacterial strains) or with individual bacterial strains *(Pseudomonas* sp. or *Acinetobacter* sp.) selected for this study. The experiment was conducted for 2 weeks, in static and dark conditions. Sediment samples were collected for high-throughput sequencing of the V4-V5 hypervariable region of the16S rRNA gene by Illumina MiSeq platform; water and sediment samples were also collected for drug analysis. Nutrient levels in all treatments were also assessed.

The presence of the pharmaceutical had a significant effect on microbial community structure, over which bioaugmentation process did not display significant changes. Low removal of paroxetine in all bioaugmented treatments was observed, in which the abiotic removal was the main removal mechanism. Nutrient concentration in all bioaugmented microcosms were close to the detection limit, indicating that nutrients from natural environment were not enough to stimulate the biodegradation process, affecting the removal efficiency. Further experiments should be conducted to optimize and develop a nutrient cocktail to stimulate the community as well as to optimize the bacterial amount that is necessary to accomplish a complete degradation of paroxetine in natural media.

4.2 Introduction

Estuaries are complex and sensitive ecosystems that are very important for different living organisms. Their unique physical-chemical conditions and strong environmental gradients, provided by the mixture of river and oceanic water intrusion, allow the establishment of extremely diverse organisms and promote vital relations with other ecosystems (Ferreira et al., 2019; Sun et al., 2012). Yet, these ecosystems are under a high anthropogenic pressure, due to the human development in the coastal and estuarine areas (Omar et al., 2019), being the ultimate receptacles for organic and inorganic pollutants (Pan & Wang, 2012). Contamination of estuarine ecosystems by different contaminants, such as pharmaceuticals, metals and hydrocarbons has been widely reported (Oliva et al., 2015; Omar et al., 2019; Yao et al., 2016). Detection of these pollutants in estuarine areas has raised concern as their presence can cause deleterious effects on the living organisms, disturbances in the ecosystem and be a threat to human health (Pan & Wang, 2012; Sun et al., 2012). From a wide range of contaminants found in estuarine ecosystems, pharmaceuticals and their active metabolites are among the most concerning pollutants (Omar et al., 2019).

Consumption of pharmaceuticals have been increasing through the years, as the population continues to growth, medical science continues to evolve, and different diseases continues to challenge and threaten the human society. As a result, pharmaceuticals have been released to the environment, mostly due to industrial activity and human excretion of parent molecule or metabolites, being concentrated mostly in the wastewater treatment plants (Fekadu et al., 2019). The effects of these pollutants in the natural ecosystems and living organisms have been addressed in the last years, and it has been proven that different classes of pharmaceuticals can exert different effects on the ecosystems and on organism's welfare. Endocrine disruption, significant shifts of microbial dynamics (that can influence the ecosystems function) and appearance of resistant genes are examples of negative effects that have been associated to the presence of pharmaceuticals in the environment (Alvarino et al., 2014a; Krzeminski et al., 2019; Luo et al., 2014).

Bioremediation, a sustainable and eco-friendly technology, has arisen in the last years as a promising technology to recover different contaminated environments. This technology relies on the metabolic capability and diverse catabolic activities of microorganisms, due to the presence of specific enzymes and catabolic genes (Tyagi et al., 2011), to partially/completely degrade certain pollutants (Roy et al., 2018). In addition, in the presence of toxic pollutants, several microorganisms have the ability to modify the

cellular membrane, to preserve the necessary biological functions, in order to adapt to the new environment (Tyagi et al., 2011).

Different bioremediation approaches can be used: biostimulation, bioaugmentation or a combination of both. Biostimulation intends to stimulate the natural degrading community, with a proper nutrient addition, by using inorganic nutrients (mainly nitrogen and phosphorus), considering the demand of C:N:P rations that are needed to lead to the pollutant's degradation (Roy et al., 2018)**.** In the other hand, bioaugmentation involves the addition of key degrading microorganisms to the contaminated site, enhancing the biodegradation of the target compound (Abed et al., 2014; Silva et al., 2009). Bioaugmentation is suitable to be applied in contaminated sites where the native microorganisms with degradative potential are insufficient or does not have the catabolic pathways for the degradation of the pollutant or even if the concentration of the pollutant is not enough to induce the catabolic enzymes (Cycoń et al., 2017; Żur et al., 2020). Bioaugmentation can be performed using autochthonous or exogenous microorganisms (Suja et al., 2014). Autochthonous microorganisms can be advantageous as the native microorganisms can adapt more easily to the natural environment (Silva et al., 2009), preserving the natural community composition of the affected site. Exogenous microorganisms may compete with natural community for the acquisition of nutrients and carbon source and they can overtake the receiving environment (Nwankwegu & Onwosi, 2017; Żur et al., 2020), disrupting the natural status.

Autochthone bioaugmentation can be applied using single bacterial strains, bacterial consortium, fungal consortium and mixed consortium (Cycoń et al., 2017). The success of bioaugmentation strategies relies on a suitable selection of microorganisms regarding their ability to degrade the pollutant by total mineralization or into nontoxic metabolites (Silva et al., 2009) and, in the ability of the selected microorganism to stablish and survive in the contaminated site (Mrozik & Piotrowska-Seget, 2010; Vuković et al., 2019). Bioaugmentation process can be inhibited or diminish by improper conditions such as high pollutant concentration, pH, organic matter content, moisture, temperature, availability of electron donor and/or acceptor, and by unbalance nutrient levels, more specifically, phosphorus and nitrogen (Mrozik & Piotrowska-Seget, 2010; Roy et al., 2018; Smith et al., 2015).

Studies have been performed using different bacterial strains or bacterial consortium to degrade pharmaceuticals (Deng et al., 2018; Duarte et al., 2019; Reis et al., 2014). However, most of these studies are performed and tested in synthetic media, without the real constrains that can appear in natural matrix media. In addition, assessment of possible

impacts caused by bioaugmentation in the natural community dynamics should be addressed for a full understanding of the ecological effects of the bioremediation process.

This study evaluated the effects of bioaugmentation process, with native microorganisms, on the dynamics of estuarine natural community, through the amplification and the sequencing of (V4-V5) 16S rRNA gene fragment by Illumina MiSeq platform. In addition, it was also evaluated the potential of those microorganisms to degrade paroxetine in natural media experiments. The presence of the bioaugmented microorganisms was followed throughout of the experiment by culture dependent methods. The paroxetine degrading bacterial consortium used for bioaugmentation was obtained from an estuarine sediment inoculum in a previous work, were it displayed a removal efficiency higher than 97% in synthetic culture media (mineral-salts medium (MM)) (results from chapter 2 and 3). Two bacterial strains from the bacterial consortium, *Pseudomonas* sp. and *Acinetobacter* sp., were also chosen to perform the biodegradation experiment with single strains, as both strains alone presented, in preliminary tests, removal of paroxetine between 68% and 84%, and defluorination of paroxetine between 65% and 78% (chapter 3).

4.3 Methodology

4.3.1 Reagents and materials

Paroxetine HCl was acquired from Enzo Life Sciences. Methanol, acetonitrile and formic acid (98%) were obtained from Sigma-Aldrich, in which all remaining reagents were analytical grade or likewise.

For this experiment, all material was decontaminated and/or sterilized to avoid external contaminations (both chemical and biological). Biological decontamination was accessed by autoclaving the material (120°C, 20 min) and chemical decontamination was performed by emerging the material in a chloride acid bath (10% (v/v)) for 24h, being then washed in deionized water.

4.3.2 Sampling

Sediment and water samples were collected in the Douro Estuary (41°08'18.0"N 8°39'28.9"W), at low tide, in March of 2019. A portion of water was collected and stored at -20ºC for further pharmaceuticals analysis and, a portion of sediment was collected and stored at -20ºC, for the pharmaceutical determination and characterization of the natural community.

4.3.3 Biodegradation experiments in natural media

Estuarine water and sediment were used in this experiment as representatives of natural ecosystem. Microcosms were assembled, in triplicates, half containing 50 mL of estuarine water and other half with 40 mL of estuarine water and 10 g of homogenized sediment. Then, microcosms were inoculated with the selected bacterial strains, *Acinetobacter* sp. and *Pseudomonas* sp. or with the bacterial consortium, following the treatment sequence as described in Figure 30. Bacterial strains and bacterial consortium (Table 5) used in this experiment were obtained in a previous study (Duarte et al., 2019; Fernandes et al., 2020), from an enrichment process with estuarine sediment sample collected in Douro Estuary. Bacterial inoculum for the consortium was prepared by suspending, in sterile saline solution (0.85%), equal proportions of biomass for each bacterial strain into the solution. For each bacterial strain, several lops of biomass of each were suspended in sterile saline solution (0.85%). Microcosms were then bioaugmented with the bacterial inoculum (PRX Mx) or bacterial strains (PRX Ac and PRX Ps) to an optical density c.a 0.1. Microcosms were then spiked with 1 mg L^{-1} of paroxetine and were incubated in dark conditions, at 21ºC (room temperature), under static conditions. Controls doped with 1 mg L^{-1} of paroxetine were assembled for both matrices, to evaluate the effects of paroxetine in the natural community as well the natural attenuation of paroxetine by the same community. In addition, abiotic controls (PRX Cte) with sterile estuarine water/ sterile water and sediment were assembled and doped also with 1 mg L^{-1} of paroxetine. Controls only with estuarine water and sediment (Ct) were performed to evaluate the natural shifts in the community imposed by laboratory conditions.

The experiment was conducted for 2 weeks. Each week, water samples from all microcosms were collected for drug analysis and for the isolation of bioaugmented bacterial strains by culture dependent methods. At the end of the experiment, portion of water samples from all microcosms were collected and filtrated with cellulose acetate syringe filters, 0.2 µm, for further nutrients analysis. Portion of sediment was collected for microbial community characterization and for drug analysis.

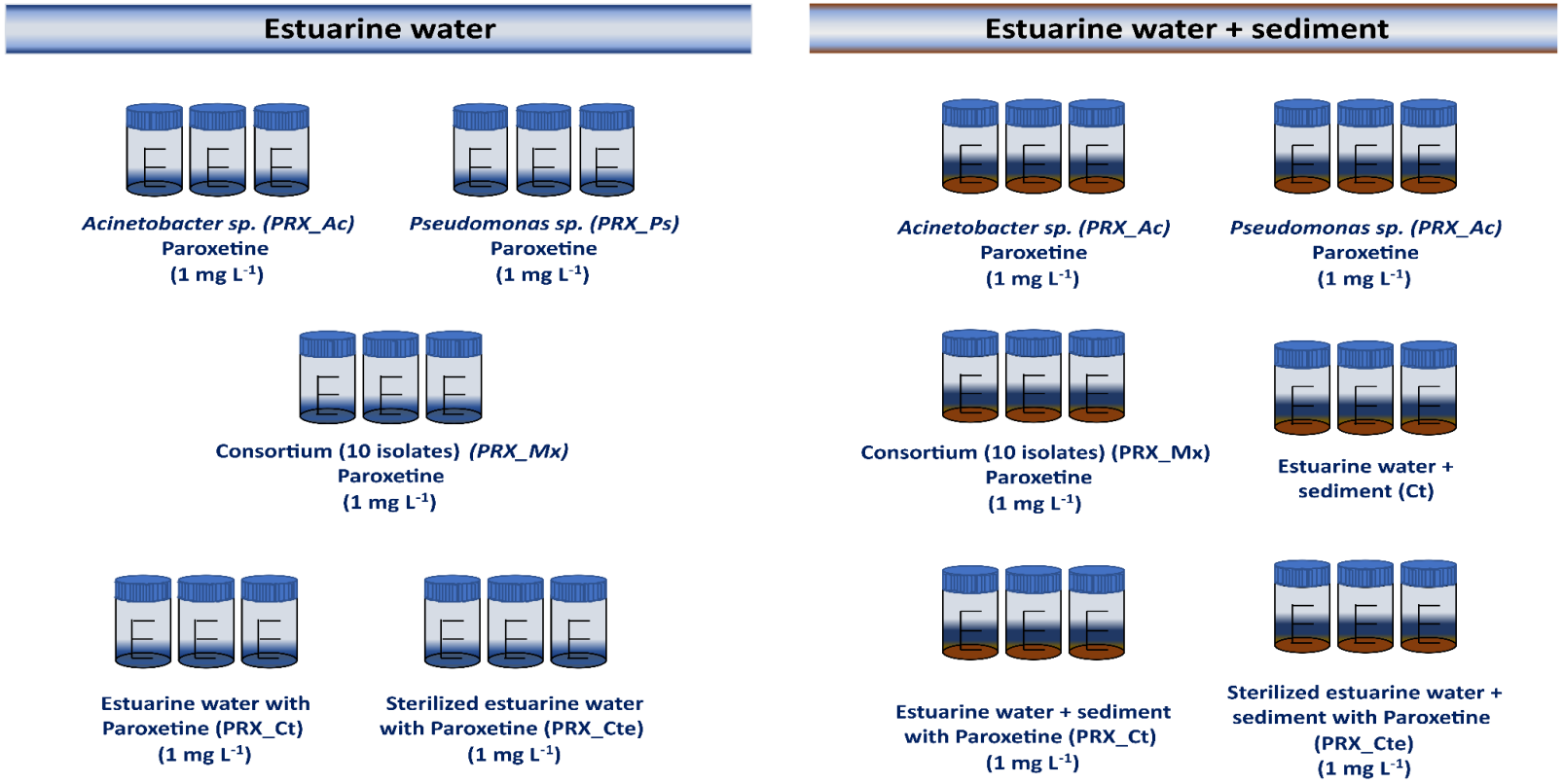

Figure 30 - Experimental design of biodegradation experiments in natural media (estuarine water and sediment). Ct - Control; PRX Cte – abiotic control; PRX Ct – control with paroxetine; PRX Ac – microcosms bioaugmented with *Acinetobacter* DPS 5; PRX Ps – microcosms bioaugmented with *Pseudomonas* DPS 10; PRX Mx, - Microcosms bioaugmented with DPS consortium. All microcosms with the PRX stands for microcosms exposed to paroxetine.

Table 5 - Taxonomic identification of bacterial isolates used in the biodegradation experiments. PCA –Plate Count Agar; MM - Mineral-salts Medium. PRX Ac – microcosms bioaugmented with *Acinetobacter* DPS 5, PRX Ps – microcosms bioaugmented with *Pseudomonas* DPS 10, PRX Mx, - Microcosms bioaugmented with DPS consortium Adapted from Fernandes et al. (2020), chapter 2.

	Bacterial strains code	**Experimental code**	**Isolation medium**	**Taxonomic identification**	**GenBank accession number**
Bacterial strains	DPS 5	PRX Ac	PCA	*Acinetobacter* sp.	MN128733
	DPS 10	PRX Ps	MM with Prx	*Pseudomonas* sp.	MN128763
Bacterial Consortium	DPS 1	PRX Mx	MM with Prx	*Pseudomonas* sp.	MN128762
	DPS 2	PRX Mx	MM with Prx	*Bosea* sp.	MN128739
	DPS 3	PRX Mx	PCA	*Shewanella* sp.	MN128776
	DPS 4	PRX Mx	PCA	*Chitinophagaceae bacterium*	MN128745
	DPS 5	PRX Mx	PCA	*Acinetobacter* sp.	MN128733
	DPS 6	PRX Mx	PCA	*Pseudomonas* sp.	MN128764
	DPS 7	PRX Mx	PCA	*Bosea* sp.	MN128740
	DPS 8	PRX Mx	PCA	*Leadbetterella* sp.	MN128750
	DPS 9	PRX Mx	PCA	*Microbacterium oxydans*	MN128754
	DPS 10	PRX Mx	MM with Prx	*Pseudomonas* sp.	MN128763

4.3.4 Isolation of bioaugmented bacterial strains and taxonomic identification by sanger sequencing 16S rRNA gene

An important feature of the bioaugmentation process is the adaptation and survival of microbial inocula on the affected site. So, it should be monitored the presence of the bioaugmented strains in the media. For that, culture dependent methods were used to access the presence of the bacterial strains in each bioaugmented microcosms. At each week, samples from each bioaugmented microcosms (PRX Ac, PRX Ps and PRX Mx) were spread in several tenfold dilutions (up to 10^{-5}) onto plate count agar (PCA) and marine agar (MA). PCA plates were incubated at 28°C during 48h and MA plates during 96h. All bacterial colonies with different morphologies were visually identified, purified, and finally collected into sterile tubes containing 100 µL of a sterile TE buffer (10 mM Tris; 1 mM EDTA; pH = 7.5) for further DNA extraction. DNA was extracted using the E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA), following the protocol provided by the manufacture. Taxonomic identification was performed through 16S rRNA gene sequence

analysis. Extracted DNA was amplified using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991) for the hypervariable regions V1–V9 of the 16S rRNA gene. Polymerase chain reaction (PCR) was performed as previously described (Fernandes et al., 2020), PCR products were quantified using Qubit® dsDNA HS Assay Kit and Qubit 3.0 fluorometer (Invitrogen), and purification and sequencing was performed by GenCore, I3S (Instituto de Investigação e Inovação em Saúde), Porto, Portugal. Sequences were analysed using Geneious 11.1.4 software (Biomatters Ltd, Auckland, New Zealand). The consensus sequences were submitted to GenBank for taxonomic identification (Nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi); using two different databases as reference: nucleotide collection and 16S ribosomal RNA sequences. EzBioCloud (Yoon et al., 2017) and Ribosomal Database Project (RDP, (Cole et al., 2014)) databases were used to confirm the results obtained in NCBI databases. All the isolated bacterial strains with the same taxonomic identification as well as the bioaugmented strain were aligned using MUSCLE from the Geneious software package to evaluate the similarity among them (when a similarity of 99.9 to 100% was obtained, it was considered that bioaugmented strain (s) were recovered).

4.3.5 Microbial community structure

Environmental DNA was extracted from 1.0 g of homogenised sediment using DNeasy PowerSoil Kit (Qiagen, GmbH, Hilden, Germany), following the protocol provided by the manufacture and quantified using Qubit® dsDNA HS Assay Kit and Qubit 3.0 fluorometer (Invitrogen). Samples were then prepared for the amplification of 16S rRNA gene fragment (hypervariable V4-V5 region; ≈412 bp) in order to characterize the microbial communities by Next Generation Sequencing (NGS). The extracted DNA was amplified and further reamplified in a limited-cycle PCR reaction (to add sequencing adapters and dual indexes), using primers set 515F-Y (5'- GTGYCAGCMGCCGCGGTAA -3') and 926R (5'-CCGYCAATTYMTTTRAGTTT -3') (Parada et al., 2016) by the Genoinseq company (Cantanhede, Portugal). For the first PCR reaction, KAPA HiFi HotStart PCR Kit was used accordingly to the manufacturer protocol. A reaction of 25 µL were performed by adding 0.3 µM of each primer (515F-Y and 926R) and 12.5 ng of template DNA. The PCR program started with the denaturation at 95ºC during 3 min, followed by 25 cycles of 98ºC for 20s, 50ºC for 30s and 72ºC for 30s, with a final extension at 72ºC for 5 min. In the second PCR reaction, indexes and sequencing adapters to both ends of the target region, amplified before, were added, following the recommendations of the manufacture (Illumina Inc.,

2013). For both PCR reactions, negative controls were performed. PCR products were purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, following the instructions provided by the manufacture (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal). Raw sequence data was pre-processed at Genoinseq (Cantanhede, Portugal). Briefly, the reads were extracted in fastq format from the Illumina MiSeq® System. The sequencing adapters, reads with less than 150 bp, trim based, and reads with an average quality lower than Q25 in a window of 5 bases, were removed using PRINSEQ version 0.20.4 (Schmieder & Edwards, 2011). Forward and reverse reads were merged by overlapping paired-end reads with AdapterRemoval version 2.1.5 (Schubert et al., 2016), using default parameters.

4.3.6 Paroxetine determination

4.3.6.1 Water samples

Paroxetine in water samples was analysed by high-performance liquid chromatography (HPLC), after a pre-treatment by solid – phase extraction (SPE) to remove the main interferants. SPE procedure was adapted from Cavenati et al. (2012). Briefly, 1 mL of samples was diluted in 14 mL of deionized water. Oasis HLB 30 µm, 3 cc, 60 mg Cartridge (Waters Corporation, Milford, MC, USA) were pre-conditioned with 5 mL of methanol followed by 5 mL of deionized water, using a vacuum manifold system (Supelco, Spain) coupled to a vacuum pump. Then, samples (15 mL) were passed through the pre-conditioned cartridges followed by 5 mL of methanol/water mixture (5:95 v/v). Loaded cartridges were dried under vacuum conditions for 30 min and eluted with 1 mL of a methanol/formic acid mixture (95:5 v/v). The eluted solution was evaporated to dryness under a gentile nitrogen stream at 30°C. In the end, residues were dissolved in 1.0 mL of mobile phase water/formic acid (99:1 v/v).

The final sample was analyzed in a HPLC Beckam Coulter equipment (System Gold) equipped with a diode array detector (module 168), an automatic sampler (module 508) and SunShell C18 2,6um 100mm X 4,6mm ID column (Chromanik Technologies, Japan), using a gradient of two mobile phases (water/formic acid (99:1 v/v) and acetonitrile, both previously degassed in an ultrasonic bath) as described in (Duarte et al., 2019). Quantification of each compound was obtained through external calibration with aqueous standard solutions (prepared in mobile phase water/formic acid (99:1 v/v), ranging between 0.1 to 1 mg L^{-1} of paroxetine.

4.3.6.2 Sediment samples

Paroxetine concentration, in initial sediments and in sediments collected after the experiment, was determined by performing two sequential extractions. In detail, 1 g of lyophilized and homogenized sediment was weight in amber vials. Then, 5 mL of methanol /ammonia (95:5 v/v) mixture was added to the sediment and the vials were placed in an ultrasonic bath (Transsonic 460/H), using a foam support, for 15 min. After that, samples were centrifuged (Selecta Mixtasel) at 2500 rpm for 5 min. The supernatant was transferred to another amber vial and the same amount of methanol/ammonia solution was added to the remaining sediment. The same procedure described before was applied. The two supernatants were combined and evaporated to dryness under a gentile nitrogen stream at 30°C. The residues were dissolved in 1.0 mL of mobile phase water/formic acid (99:1 v/v). The solution was filtered using MS Glass Fiber Syringe filters, (1.0 um, 25mm) to clean and prepare the sample for HPLC analysis. Paroxetine concentration was measured by HPLC as described in the section 4.3.6.1.

4.3.6.3 Nutrient Analysis

Nutrient levels, in the initial estuarine water and water samples at the end of the experiment, were analysed by spectrophotometry (VWR V-1200 spectrophotometer). To mimic the nutrients available in the initial sediment, initial estuarine water was mixed with a portion of sediment, in the same proportions represented in the microcosms. The solution was shaken and filtered as described in section 4.3.3. Ammonium concentration was determined using the Grasshoff & Johannsen (1972) method, an adaptation of Koroleff (1970). Nitrate and phosphate ions concentration were quantified using the methods described by Grasshoff et al. (2009). Nitrate ion concentration was measured using the adaptation of the spongy cadmium reduction technique according to Jones (1984).

4.3.7 Data Analysis

4.3.7.1 Microbial community analysis

The methodology used in this study was first reported by Ionescu et al. (2012) and Klindworth et al. (2013). Filtered merged amplicons received by the sequencing company in fastq format were then converted into fasta format by Mothur software (mothur v.1.43.0; (Schloss et al., 2009)). Sequences were processed using Next-Generation Sequencing (NGS) analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) (Quast et al., 2012). Each read was aligned against the SILVA SSU rRNA SEED, using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008)) (Pruesse et al., 2012)

and quality check was controlled (Quast et al., 2012). Reads with more than 2% of ambiguities or 2% of homopolymers and reads with less than 50 aligned nucleotides were removed. Artefacts reads with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA) and putative contaminations were also removed. Afterward, dereplication and clustering was performed using CD-HIT-EST (version 3.1.2; http://www.bioinformatics.org/cd-hit/) (Li & Godzik, 2006) using as parameter the *accurate mode* (identity criteria of 1.00), ignoring overhangs (identity criteria of 0.98). Specifically, identical reads were identified (dereplication), unique reads were clustered (OTUs) and reference read of each OTU was classified. A local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 132; https://www.arb-silva.de/) using blastn (version 2.2.30+; https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed for the taxonomic classification, using standard settings (Camacho et al., 2009). Each OTU was mapped against all reads that were assigned to the respective OTU. All reads with low BLAST hits (lower than 93%) or without BLAST hit, remained unclassified.

Undesirable lineages, “Chloroplast”, “Mitochondria” and “Eukaryota” were excluded from the dataset. Data resulting from SILVAngs analysis were imported to R software (version 4.0.2; https://www.r-project.org/) to analyse the diversity, taxonomic composition of communities in the different sediment samples and Multinomial Species Classification Method (CLAM).

Taxonomic profile of the prokaryotic community, alpha and beta diversity analysis were performed using phyloseq package (version 1.32.0) from R software. For alpha diversity, two different metrics were calculated, OTUs richness (number of clustered OTUs) and Shannon diversity. For beta diversity, non-metric multidimensional scaling (NMDS) analysis, based on Bray–Curtis dissimilarities, was performed. Multivariate analysis using ANOSIM function (analysis of similarities) from vegan package (version 2.5.6) (R software) were performed to assess statistical shifts on the community by bioaugmentation process, presence of pharmaceutical and laboratory conditions.

4.3.7.2 Multinomial Species Classification Method (CLAM)

In order to assess the species affinities for the two distinct experimental conditions (control with paroxetine (PRX Ct) and control without paroxetine (Ct)), OTUs were classified as “generalists” and “specialists” performing the “clamtest” function in the vegan package from R software. Multinomial Species Classification Method (CLAM) (Chazdon et al., 2011) uses a multinomial model based of relative abundance of estimated OTUs for two distinct conditions. To perform the analysis, a clean OTU table was used as input data to classify

generalists and specialists in two different treatments. A conservative threshold based on the super-majority rule (K = 2/3, P = 0.005) was applied in order to obtain a robust statistical classification of specialists or generalists' microorganisms among the tested groups without excluding rare species a priori that are present in the community. The clam multinomial model classifies taxa into one of four groups: (1) "generalist"; (2) "Ct specialist"; (3) "PRX Ct specialist"; and (4) "too rare to classify" with confidence. Clam output from R was transferred for an Excel sheet with the taxonomic information. Then, a match among taxonomic rank and each classified specialist and generalist OTU was performed using the function VLOOKUP from excel.

4.3.7.3 NGS dataset *vs* isolated bioaugmented strains

A deeper analysis was also performed running a local BLAST search of the NGS environmental sequences obtained through Illumina MiSeq®, against private sequences of the isolated strains used in this study (accession numbers available in chapter 2, Table 1). For these purpose, V4-V5-16S rRNA raw sequences provided by the sequencing company (Genoinseq), were pre-processed using mothur pipeline. Individually, the forward and reverse reads of each library were joined from raw Illumina fastq files. Merged reads with ambiguities (also known as N) and shorter than 300 bp were excluded as well as the ones with homopolymers (n>8). The remaining sequences were dereplicated (based on 100% similarity). After dereplication step, chimeric sequences were identified by denovo and removed with UCHIME (Edgar et al., 2011).

Taxonomic assignment of the unique reads (obtained after chimera removing) was performed using standalone BLAST in BLAST+ suite (Altschul et al., 1990; Camacho et al., 2009) against a custom reference database which contained the sequences of the 16 rRNA gene (V4-V5 regions) from the bioaugmented isolated strains used in this study. The purpose of this analysis was to evaluate the presence of the specific bioaugmented strains in the respective treatments results, obtained by culture independent methods (NGS). To validate this analysis results, a deeper check of the consensus sequence was performed. Briefly, each V4-V5 16S rRNA consensus sequence of bioaugmented strains was aligned with the five closest sequences (obtained from nucleotide NCBI BLAST (rRNA/ITS DATABASES - 16S ribosomal RNA sequences, https://blast.ncbi.nlm.nih.gov/Blast.cg), in order to verify if they shared the same V4-V5 region with one or more sequences present in NCBI database. Alignments of 100% similarity, meaning that the type strains and bioaugmented strain have complete similarity in the target V4-V5 hypervariable regions, were an indicator that the barcoding marker is identic in one or more species and thus, it is

unable to discriminate species that are close relatives. In this case, the strain discrimination failed (strains cannot be distinguished), and the results obtained by the performed local blast cannot be validated.

4.3.7.4 Pharmaceuticals removal and nutrient levels

For statistical analysis, triplicates were analysed and treated independently, and the mean values and respective standard deviations were calculated. The statistical differences for removal efficiency and nutrients parameters were analysed using STATISTIC software version 12 (StatSoft, Inc., 2013). Significant differences among treatments and control (p-value below or equal to 0.05 (confidence level of 95%)), were assessed through a parametric Student's t-test, using mean values and corresponding standard deviations of the replicates (n=3).

4.4 Results

4.4.1 Microbial Community Dynamics

High throughput sequencing of the whole community was performed with the objective to characterize the community structure associated with different factors, such as experimental conditions, addition of paroxetine or bioaugmentation. A total of 1.077.107 of V4-V5 16S rRNA gene sequences were generated by Illumina MiSeq sequencing for all the 18 samples (including the initial sediment) which decreased to a total of 1.063.451 after the quality filtering performed by the sequencing company. A total of 970.578 merged reads were processed by SILVAngs analysis pipeline. From those, 779.654 were classified, 163.513 (16.85%) sequences were rejected (after Silva NGS treatment) and 27.411 (2.82%) remained classified as ‘No relative’ reads (without any close relatives).

4.4.2 Alpha Diversity

To evaluate alpha diversity, two different metrics were calculated: observed operational taxonomic units (OTUs) and Shannon diversity (Figure 31). The NGS results of the V4-V5 16S rRNA gene amplicons revealed that the addition of paroxetine and the bioaugmentation process led to a substantial decrease on the richness (observed OTUs) and diversity (Shannon) of the microbial community present in the estuarine sediment. For the observed OTUs (Figure 31), initial sediment (IS) and control without paroxetine (Ct) displayed higher OTU values compared with the treatments with paroxetine and bioaugmentation ($p<0.05$). In addition, control with paroxetine (PRX Ct) and treatment bioaugmented with the consortium (PRX Mx) displayed lower OTU values comparing with

treatments bioaugmented with the *Acinetobacter* (PRX Ac) or *Pseudomonas* (PRX Ps). Initial sediment (IS) and control without paroxetine (Ct) displayed higher diversity (Figure 31) comparing with the treatments with paroxetine and bioaugmentation ($p<0.05$). Shannon index also showed that treatment with *Pseudomonas* sp. (PRX Ps) displayed lower values of diversity ($p<0.05$), comparing with the other bioaugmented treatments and control with paroxetine (PRX Ct). In both metrics, no clear pattern was observed regarding the influence of bioaugmentation in the community. Otherwise, the addition of pharmaceutical displayed a clear shift in prokaryotic diversity and observed OTUs.

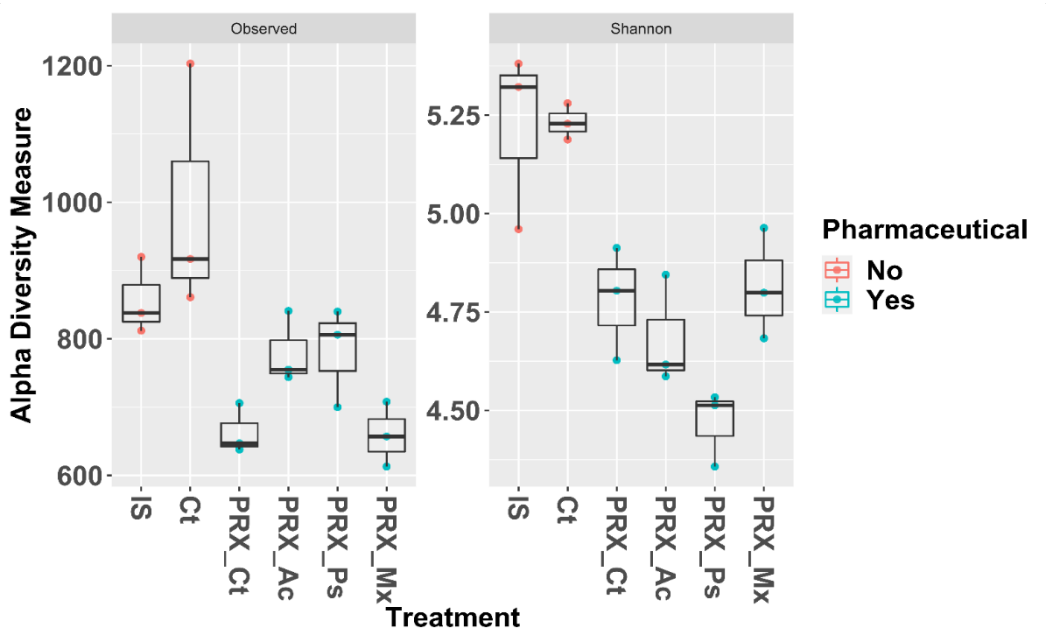

Figure 31 - Alpha diversity (observed OTUs and Shannon diversity) in the initial sediments and sediments from each treatment, after two weeks of experiment. IS – Initial sediment; Ct -Control (natural community), PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with *Acinetobacter* DPS 5; PRX Ps – Bioaugmented with *Pseudomonas* DPS 10; PRX Mx– Bioaugmented with bacterial consortium (DPS).

4.4.3 Microbial community structure - Beta diversity

Prokaryotic communities are sensitive to the presence of contaminants, therefore, shifts in function and structure of the natural community can occur. Moreover, despite the selection and application of autochthonous microorganisms, the addition of bacterial cocktails to the natural community can change the community dynamics. Thus, to evaluate the potential effects of both contaminant and bioremediation technology in the community, a Nonmetric Multidimensional Scaling analysis (NMDS) was performed, based on Bray Curtis dissimilarities. NMDS analysis (Figure 32) clearly showed two main groups: one

group constituted by the initial sediment (IS) and the control without paroxetine (Ct) and, a second one grouping the control with paroxetine (PRX Ct) and all bioaugmented treatments. These results showed that the addition of pharmaceutical changed the community structure. In addition, experimental conditions did not have a significant effect on the natural community as no clear changes were observed between the initial sediment (IS) and the experimental control (Ct).

ANOSIM analysis (Analysis of (dis)similarities, (Clarke, 1993)) was performed to unveil the factors responsible for the shaping of the community structure. ANOSIM analysis (Table 6) unveiled that the experimental conditions did not significantly affect the community structure. This led us to investigate if the community dynamics can be associated with two other factors: presence of the pharmaceutical and/or bioaugmentation addition. The presence of pharmaceutical was the factor that clearly affected the community structure (statistical value closer to 1, $p < 0.0003$). On the other hand, bioaugmentation did not have a significant effect on the shaping of the community structure.

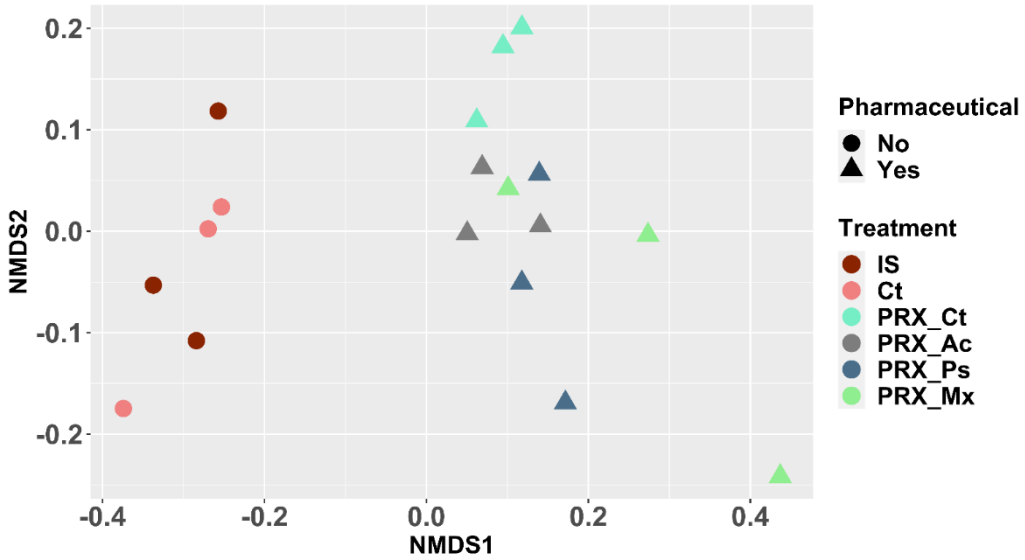

Figure 32 - Beta diversity represented by Non-metric multidimensional scaling ordination based on Bray–Curtis dissimilarities of bacterial communities in initial sediments and in each treatment after two weeks of experiment. IS – Initial sediment; Ct -Control (natural community), PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with *Acinetobacter* DPS 5; PRX Ps – Bioaugmented with *Pseudomonas* DPS 10; PRX Mx– Bioaugmented with bacterial consortium (DPS).

Table 6 - One-way ANOSIM analysis for the experimental conditions, bioaugmentation and pharmaceutical effect on the prokaryotic community structure (9999 permutations).

Factor	R^2	Pr (>F)
Experimental conditions	0.4074	0.3
Pharmaceutical	0.9338	0.0003
Bioaugmentation	0.4005	0.0032

4.4.4 Characterization of the prokaryotic communities

Taxonomic profile of the prokaryotic communities, in the initial sediment and in the different sediments after the experiment, was performed at phylum and genus level (or prior taxonomic attribution). As shown before, communities exposed to paroxetine displayed different community structure. At phylum level (Figure 33), Proteobacteria, Bacteroidetes and Planctomycetes were the most dominant phyla (relative abundance higher than 1%) in the natural community (initial sediment) and laboratorial control (control without paroxetine). For the control with paroxetine (PRX Ct) and all bioaugmented treatments, Proteobacteria, Bacteroidetes and Euryarchaeota were the most abundant phyla (relative abundance higher than 1%). Analysis to genus level was performed however, for some microorganisms, taxonomic profile was only obtained to family, order, class or, in some cases, phylum level. In the initial community and control without paroxetine (Ct) (Figure 34), the genera *Ulvibacter, Woeseia and* microorganisms belonging to Rhodobacteraceae family were the most abundant (relative abundance higher than 2%). In addition, a significant part of the community represented in both communities were assembled as “no relative”. For the control with paroxetine (PRX Ct) and for all bioaugmented treatments, microorganisms belonging to the Methylophagaceae family were the most abundant, followed by the genera *Methanococcoides* and *Methanolobus* (relative abundance higher than 2%).

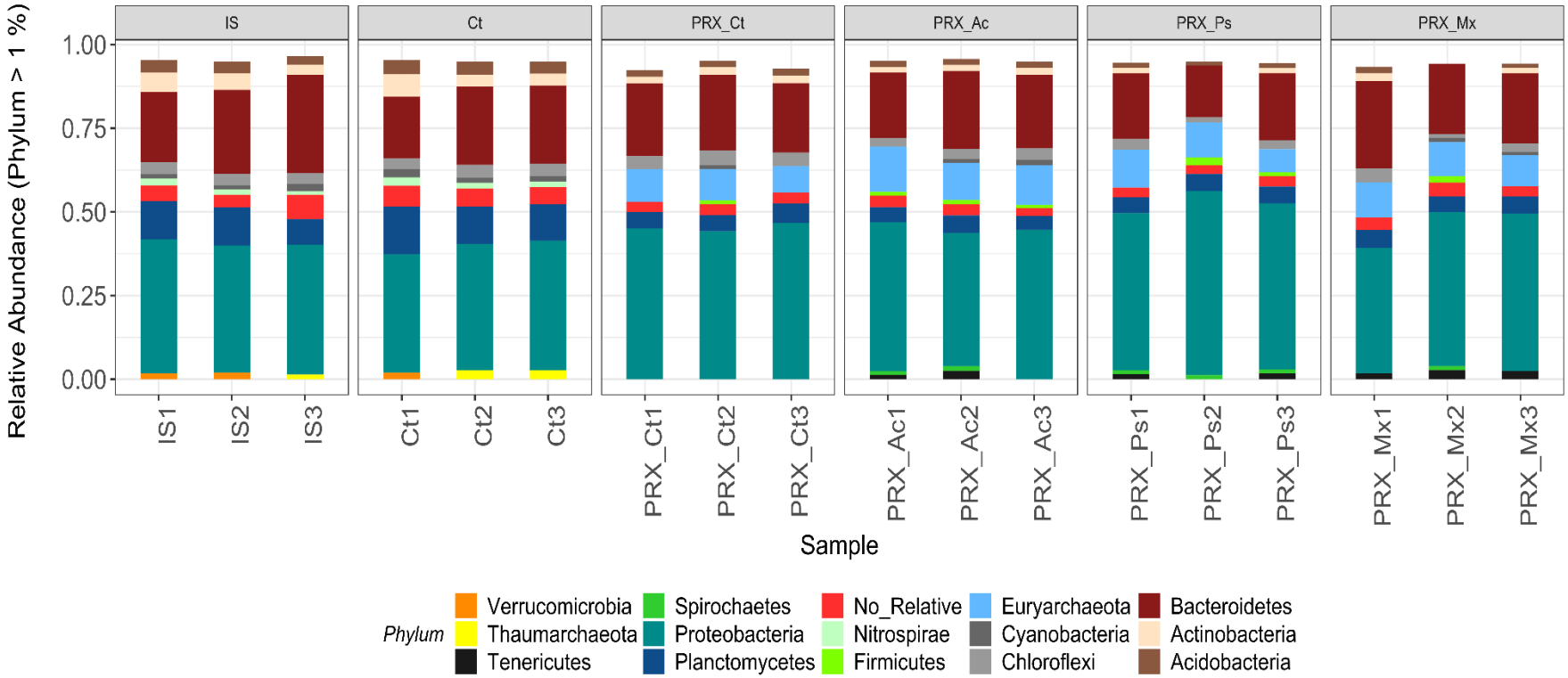

Figure 33 - Taxonomic profile at phylum level in the initial sediment and in sediments for each treatment, after 2 weeks of experiment. IS – Initial sediment; Ct -Control (natural community), PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with *Acinetobacter* DPS 5; PRX Ps – Bioaugmented with *Pseudomonas* DPS 10; PRX Mx– Bioaugmented with bacterial consortium (DPS).

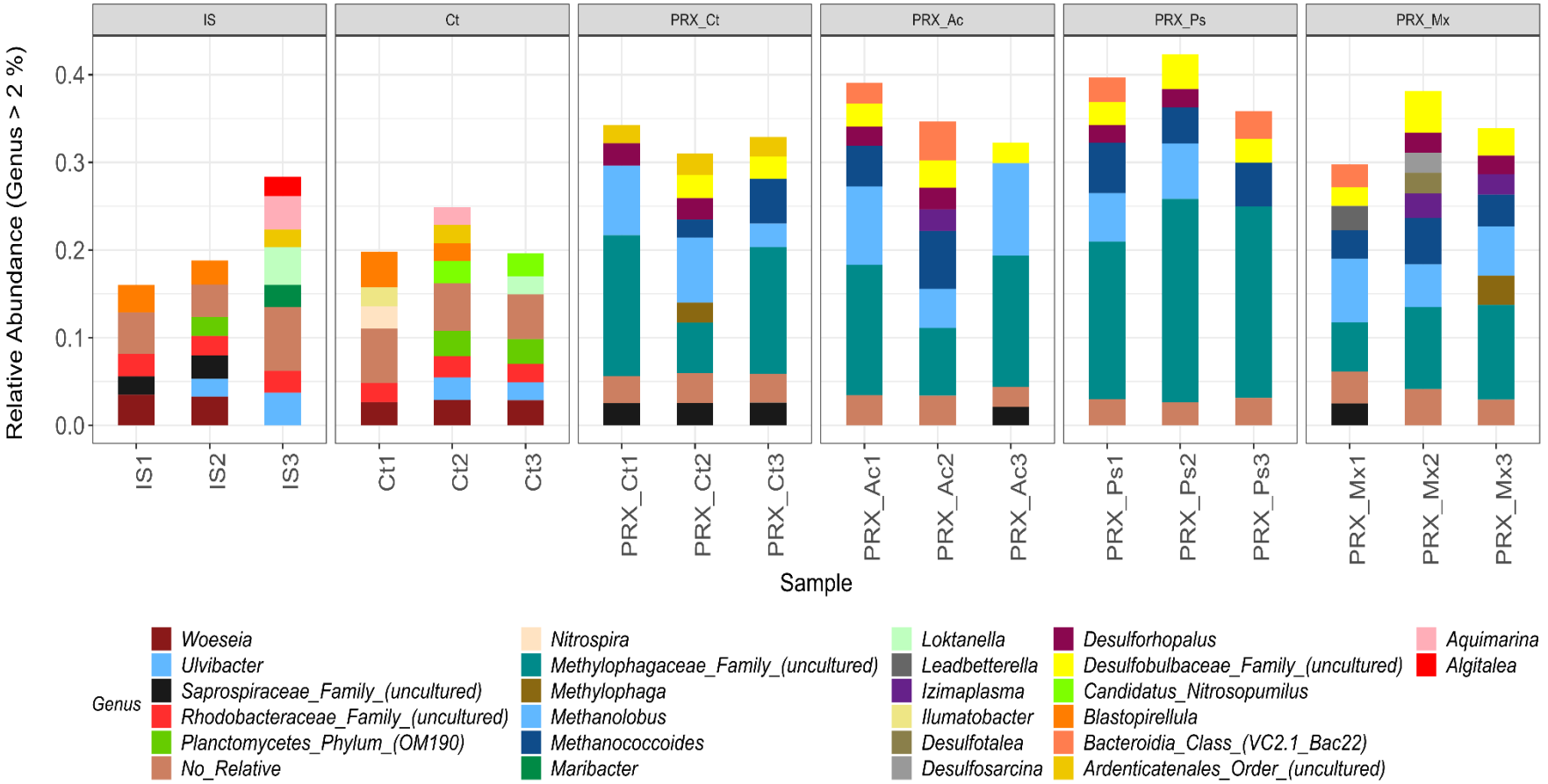

Figure 34 - Taxonomic profile at genus level in the initial sediment and in sediments after 2 weeks of each treatment after two weeks of experiment. IS – Initial sediment; Ct -Control (natural community), PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with *Acinetobacter* DPS 5; PRX Ps – Bioaugmented with *Pseudomonas* DPS10; PRX Mx– Bioaugmented with bacterial consortium (DPS).

4.4.5 Recovered bacterial strains by culture dependent and independent methods

Culture dependent and independent methods were performed to assess the presence of the bacterial strains used as bioaugmentation inocula, in the end of the experiment, in order to understand if they were able to survive within the natural community. Regarding the recovery of the bacterial strains by culture depend methods, a sample from each microcosm was spread in several tenfold dilutions (up to 10^{-5}) onto PCA and MA plates. All isolates with different morphological features were purified and identified. The isolates recovered in MA media did not grow properly and their identification was not performed. The consensus sequences obtained in this study were aligned with the strains used in the bioaugmented microcosms to assess their similarity. In the microcosms bioaugmented with *Pseudomonas* sp. (DPS 10) (Tables 7 and 8), the strain was recovered in the respective treatment (similarity of 100%), in microcosms with and without sediment. For strain *Acinetobacter* sp. (DPS 5), similarity among the recovered strain and the bioaugmented one ranged between 99.8% - 99.9%, thus, there is no assurance that the recovered strain is the one that was used in the bioaugmented process. Regarding the consortium, the strains *Bosea* sp. (DPS 2) and *Pseudomonas* (DPS 1) were recovered in the two treatments bioaugmented with the consortium, with similarities of 100% for both strains. In addition, *Pseudomonas* sp. (DPS 6) was recovered from the treatment bioaugmented with the consortium without sediment (Table 7) and the strain *Microbacterium* (DPS 9) was recovered from the treatment bioaugmented with the consortium with sediment (Table 8). *Acinetobacter* sp. (DPS 5) was found in both treatments bioaugmented with the consortium, however, as shown in the bioaugmented treatments only with the single strain, the recovered strain displayed a similarity of 99.9% with the one that was bioaugmented.

For culture independent methods, standalone BLAST in BLAST+ suite was performed, using the dataset obtained by the V4-V5 16S NGS analysis *vs* the consensus sequences of bioaugmented strains used in this study. This analysis was only performed for the microcosms assembled with mixed estuarine water and sediment. Local blast analysis (Figure 35) showed that bioaugmented strains were potentially present in the NGS respective treatments. *Pseudomonas* sp. (DPS10), recovered by culture dependent methods, was detected in the NGS respective treatment (PRX Ps) and in the consortium, presenting a 100% of similarity (DPS 10). Looking at the alignment of the V4-V5 region between the closest type strains and the bioaugmented strain, none of the type strains closest to *Pseudomonas* sp. (DPS 10) presented similarity of 100% for the target

hypervariable region, indicating that, unambiguously, this strain was present at the end of the experiment in the NGS respective treatments (V4-V5 marker region was able to discriminate this strain). *Leadbetterella* sp. (DPS 8) was also detected in the consortium results obtained by NGS (100% similarity), and also in this case no type strain presented a complete similarly for V4 – V5 region with the bioaugmented strain (again V4–V5 successful strain discrimination). For the remaining bacterial strains that constitute the consortium and were detected by the local blast in the respective NGS treatment (with 100% of identity with environmental sequences), it cannot be ensured the successful discrimination. Indeed, looking at the V4–V5 multialignment of the bioaugmented strains DPS 1, DPS 3, DPS 6, it showed that all shared 100% of similarity in V4–V5 region with the respective type strains. It makes impossible to unambiguously ensure the presence of these bioaugmented strains in the NGS respective treatments.

Gathering both methods, strain *Pseudomonas* (DPS 10) was recovered in the treatment bioaugmented with the strain alone, in microcosms assembled with mixed estuarine water and sediment, validated by both methods. Strains recovered by culture dependent method, displaying an alignment similarity of 100% can be considered as recovered. Most of these strains were not validated by culture independent methods, however, this method only analysed a specific hypervariable region.

Table 7 - Bacterial isolates recovered from microcosms without sediment. n.a – not applied. PRX Ac – Bioaugmented with *Acinetobacter* DPS 5; PRX Ps – Bioaugmented with *Pseudomonas* DPS 10; PRX Mx– Bioaugmented with bacterial consortium (DPS).

Treatment	Bioaugmented strain	Recovered strain	Pairwise Identity (%)
1st Week			
Acinetobacter (PRX Ac)	*Acinetobacter* sp. DPS 5	*Acinetobacter* sp.	99.8
Pseudomonas (PRX Ps)	*Pseudomonas* sp. DPS 10	*Pseudomonas* sp.	99.9
Consortium (PRX Mx)	*Pseudomonas* sp. DPS 1	*Pseudomonas* sp.	100
	Bosea sp. DPS 2	Not recovered	n.a
	Shewanella sp. DPS 3	Not recovered	n.a
	Chitinophagaceae bacterium DPS 4	Not recovered	n.a
	Acinetobacter sp. DPS 5	*Acinetobacter* sp.	99.9
	Pseudomonas sp. DPS 6	Not recovered	n.a
	Bosea sp. DPS 7	Not recovered	n.a
	Leadbetterella sp. DPS 8	Not recovered	n.a
	Microbacterium oxydans DPS 9	*Microbacterium oxydans*	100
	Pseudomonas sp. DPS 10	Not recovered	n.a
2nd Week			
Acinetobacter (PRX Ac)	*Acinetobacter* sp. DPS 5	*Acinetobacter* sp.	99.9
Pseudomonas (PRX Ps)	*Pseudomonas* sp. DPS 10	*Pseudomonas* sp.	100
Consortium (PRX Mx)	*Pseudomonas* sp. DPS 1	*Pseudomonas* sp.	100
	Bosea sp. DPS 2	*Bosea* sp.	100
	Shewanella sp. DPS 3	Not recovered	n.a
	Chitinophagaceae bacterium DPS 4	Not recovered	n.a
	Acinetobacter sp. DPS 5	*Acinetobacter* sp.	99.9
	Pseudomonas sp. DPS 6	*Pseudomonas* sp.	100
	Bosea sp. DPS 7	Not recovered	n.a
	Leadbetterella sp. DPS 8	Not recovered	n.a
	Microbacterium oxydans DPS 9	Not recovered	n.a
	Pseudomonas sp. DPS 10	Not recovered	n.a

Table 8 - Bacterial isolates recovered from microcosms assembled with sediment. n.a – not applied. PRX Ac – Bioaugmented with *Acinetobacter* DPS 5; PRX Ps – Bioaugmented with *Pseudomonas* DPS 10; PRX Mx– Bioaugmented with bacterial consortium (DPS).

Treatment	Bioaugmented strain	Recovered strain	Pairwise Identity (%)
1st Week			
Acinetobacter (PRX Ac)	*Acinetobacter* sp. DPS 5	*Acinetobacter* sp.	99.8
Pseudomonas (PRX Ps)	*Pseudomonas* sp. DPS 10	*Pseudomonas* sp.	100
Consortium (PRX Mx)	*Pseudomonas* sp. DPS 1	*Pseudomonas* sp.	100
	Bosea sp. DPS 2	*Bosea* sp.	100
	Shewanella sp. DPS 3	Not recovered	n.a
	Chitinophagaceae bacterium DPS 4	Not recovered	n.a
	Acinetobacter sp. DPS 5	*Acinetobacter* sp.	99.9
	Pseudomonas sp. DPS 6	Not recovered	n.a
	Bosea sp. DPS 7	Not recovered	n.a
	Leadbetterella sp. DPS 8	Not recovered	n.a
	Microbacterium oxydans DPS 9	*Microbacterium oxydans*	100
	Pseudomonas sp. DPS 10	Not recovered	n.a
2nd Week			
Acinetobacter (PRX Ac)	*Acinetobacter* sp. DPS 5	*Acinetobacter* sp.	99.9
Pseudomonas (PRX Ps)	*Pseudomonas* sp. DPS 10	*Pseudomonas* sp.	100
Consortium (PRX Mx)	*Pseudomonas* sp. DPS 1	*Pseudomonas* sp.	100
	Bosea sp. DPS 2	*Bosea* sp.	100
	Shewanella sp. DPS 3	Not recovered	n.a
	Chitinophagaceae bacterium DPS 4	Not recovered	n.a
	Acinetobacter sp. DPS 5	*Acinetobacter* sp.	99.9
	Pseudomonas sp. DPS 6	Not recovered	n.a
	Bosea sp. DPS 7	Not recovered	n.a
	Leadbetterella sp. DPS 8	Not recovered	n.a
	Microbacterium oxydans DPS 9	*Microbacterium oxydans*	100
	Pseudomonas sp. DPS 10	Not recovered	n.a

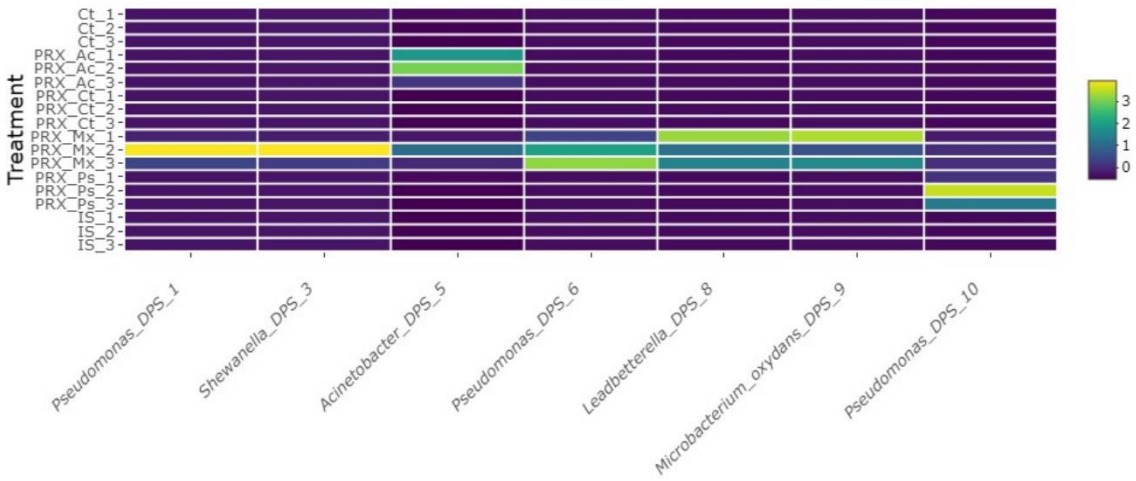

Figure 35 - Heatmap representing the presence of bioaugmented strains in each treatment, using NGS data set against the consensus sequences of the bioaugmented strains.

4.4.6 Generalists Versus Specialists: CLAM analysis

CLAM analysis was performed to identify the potential specialists in the community after the exposure to paroxetine, by testing control with paroxetine (PRX Ct) against the control without paroxetine (Ct). Of the classified OTUs, 29% were classified as “generalists” and 62% were classified as” too rare” for both treatments. In addition, 4% were classified as “specialists for the Ct treatment” and 4% were classified as “specialists for the PRX Ct treatment”, *i.e.*, taxa with a preference for one of the two environments. Analysing the clam results obtained for control without paroxetine (Figure 36), most of the OTUs classified as specialist by the algorithm belong to the phylum Proteobacteria. Species belonging to the phylum Actinobacteria, Bacteroidetes, Planctomycetes, Acidobacteria and Verrucomicrobia were also classified as specialists in the experimental control (Ct).

Concerning the control with paroxetine (Figure 37), most of the OTUs classified as specialist by clam analysis also belong to the phylum Proteobacteria. In addition, an increasing on species belonging to the phylum Bacteroidetes was observed. With the addition of paroxetine, species from Spirochaetes, Firmicutes, Epsilonbacteraeota and Euryarchaeota (Archaea) were classified as specialists. The genera *Methanococcoides* and *Methanolobus* and Methylophagaceae family (the most abundant in the control with paroxetine, Figure 34) are among the specialists (data not shown).

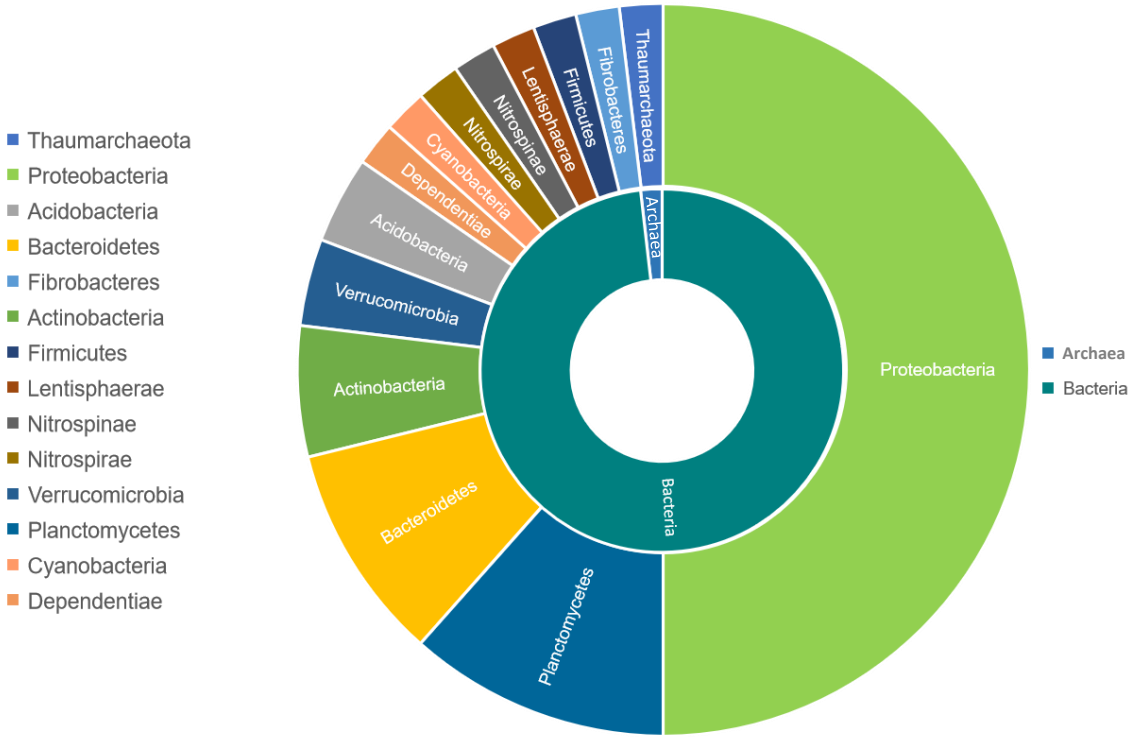

Figure 36 - Prokaryotic community classified as specialists by Multinomial Species Classification Method (CLAM) analysis in the control without paroxetine (Experimental control, Ct).

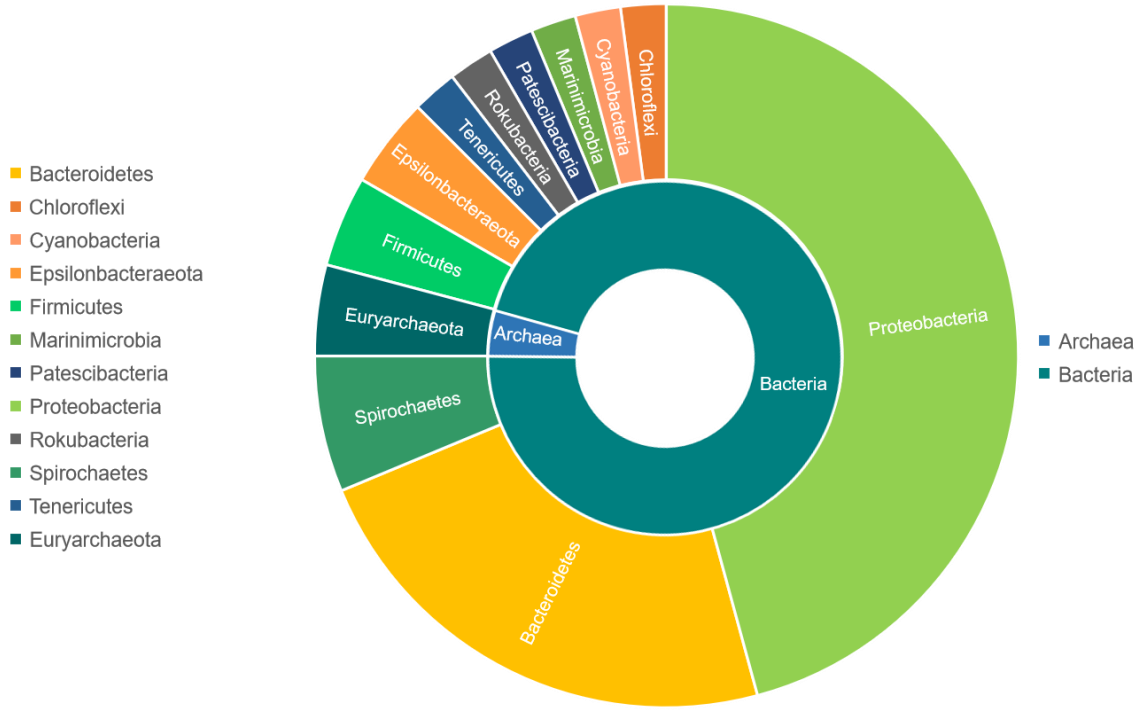

Figure 37 - Prokaryotic community classified as specialists by Multinomial Species Classification Method (CLAM) analysis in the control with paroxetine (PRX Ct).

4.4.7 Analytical Analysis

4.4.7.1 Nutrient consumption

Levels of nutrients (ammonium, nitrite, nitrate and phosphate) were determined in the initial water (IW) and water samples from all treatments. To assess the nutrient concentration in the initial sediment (IS), initial water and sediment were mixed, agitated and water was collected and filtered. In the microcosms without sediment (Figure 38), results showed that all nutrient concentrations significantly decreased in the treatments with paroxetine ($p<0.05$), including the control, comparing with the sterile control (PRX Cte) and initial water (IW). An exception for nitrite concentration was observed, which displayed a slightly variation. These results suggest that natural community consumed the nutrients in the systems in response to the addition of paroxetine. Comparing the control with paroxetine and all bioaugmented systems (Figure 38), ammonium concentration significantly decreased in bioaugmented treatments ($p<0.05$), however, in general, nitrite and nitrate concentrations significantly increased in the bioaugmented treatments ($p<0.05$), pointing that nitrification process may had occurred. In the initial water, ammonium levels were significantly higher comparing with the sterile control ($p<0.05$). These levels decreased in the sterile control followed by a significant increase of nitrate levels ($p<0.05$), indicating that ammonium was converted into nitrate. The same pattern was observed in the control with paroxetine (PRX Ct) and the bioaugmented treatments ($p<0.05$).

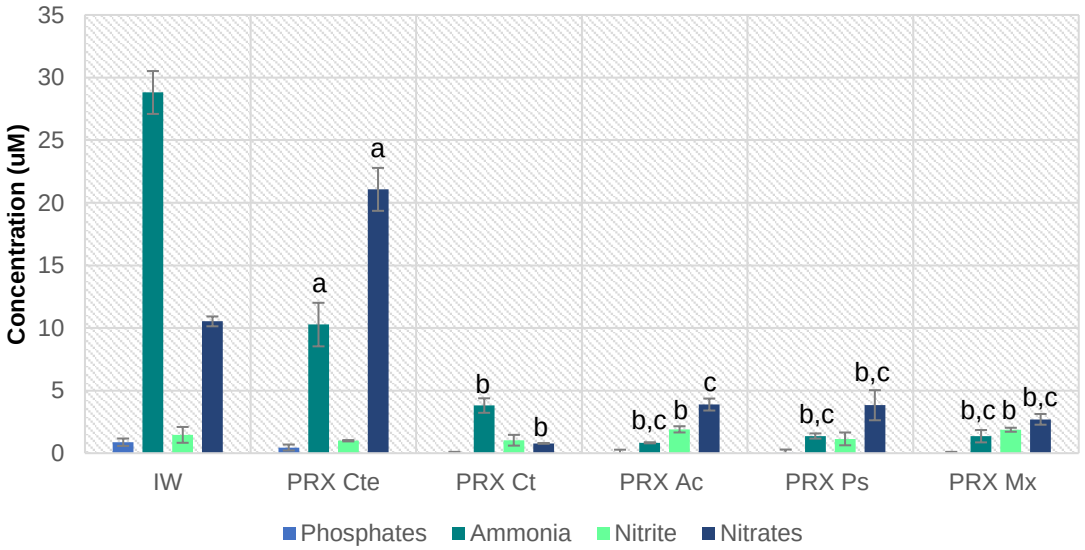

Figure 38 - Characterization of water regarding the nutrients concentrations (phosphate, ammonium, nitrite and nitrate ions (mean and standard deviation, n = 3) in the different treatments, for the microcosms without sediment. a - Significant differences among the initial water (IW) and sterile control (PRX Cte); b – Significant differences among the sterile control (PRX Cte) and biotic treatments at the same time ($p < 0.05$); c – significant differences among the control with paroxetine and bioaugmented treatments ($p<0.05$). IW – Initial water; PRX Cte - sterile control; PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with *Acinetobacter* DPS 5; PRX Ps – Bioaugmented with *Pseudomonas* DPS 10; PRX Mx– Bioaugmented with bacterial consortium.

Regarding the microcosms with sediment (Figure 39), in general, the same pattern was observed in treatments with paroxetine, in which it was observed a significant decrease of nutrient concentrations ($p<0.05$). Although, exceptions were observed for ammonium concentrations in the bioaugmented treatments, as a significant increasing of ammonium concentration was observed compared with the control with paroxetine, sterile control and initial water ($p<0.05$). As observed in the microcosms without sediment, the natural community consumed the nutrients in the systems in response to the addition of paroxetine, indicating a response of the community. With the depletion of nutrient concentration by the natural community, nutrient concentration may not be enough to stimulate the bioaugmented community.

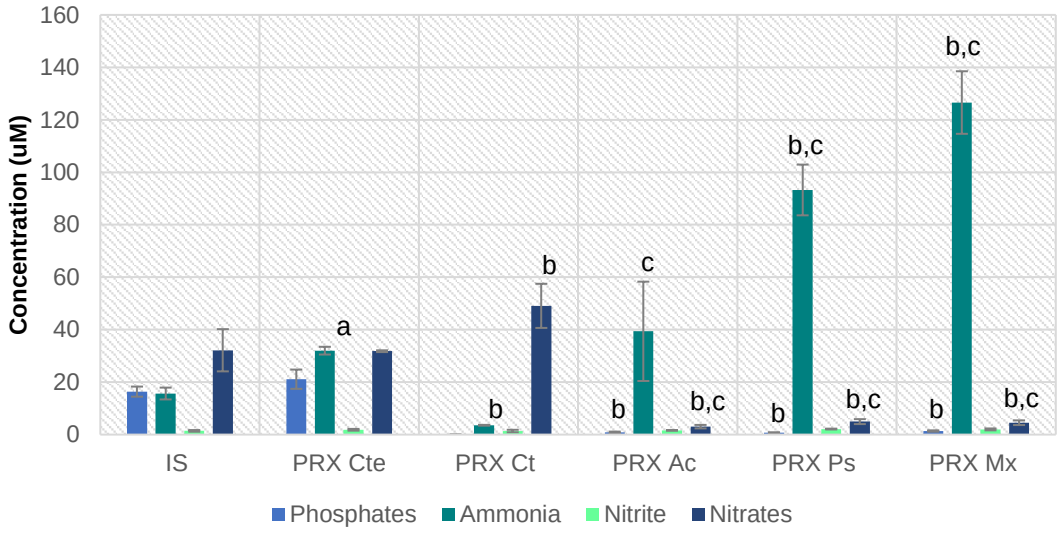

Figure 39 - Characterization of water regarding the nutrients concentrations (phosphate, ammonium, nitrite and nitrate ions (mean and standard deviation, n = 3) in the different treatments, for the microcosms with sediment. a - Significant differences among the initial water and sterile control; b – Significant differences among the sterile control (abiotic) and biotic treatments at the same time ($p < 0.05$); c – significant differences among the control with paroxetine and bioaugmented treatments ($p<0.05$). IS – Initial sediment; PRX Cte - sterile control; PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with *Acinetobacter* DPS 5; PRX Ps – Bioaugmented with *Pseudomonas* DPS 10; PRX Mx– Bioaugmented with bacterial consortium.

4.4.7.2 Paroxetine determination

4.4.7.2.1 Microcosm with and without sediment

Concentration of paroxetine was analysed in both water and sediment samples and total removal was evaluated (Figure 40). Results showed that around 70% of paroxetine was removed in all bioaugmented treatments, slightly higher compared with the abiotic control (PRX Cte, 60%) ($p< 0.05$). However, the removal observed in the bioaugmented treatments was not significant comparing with the control with paroxetine ($p>0.05$). Initial sediments displayed a concentration of paroxetine of 3.5 ug g-1 of sediment and this value was considered to assess the total removal of paroxetine.

Taking in consideration the paroxetine concentration found in the initial sediments and, comparing the results obtained for microcosms assembled only with estuarine water and the ones assembled with estuarine water and sediment, adsorption of paroxetine into the sediments appears to be one of removal mechanisms. In addition, lower concentration of paroxetine was found in the water samples from microcosms with sediment, with

paroxetine being detected in the sediments, indicating that adsorption to sediments may had occur.

Paroxetine concentration was determined in water throughout the experiment and in initial water (IW) (Figure 40) to evaluate the removal efficiency of the bioaugmentation process.

Significant removal efficiency was observed for microcosms inoculated with the strain *Acinetobacter* and with the consortium (44% in each treatment), after two weeks of experiment, comparing with sterile control and control with paroxetine. However, most of the observed removal was due to abiotic degradation (PRX Cte, 30%). In addition, natural attenuation by the natural community was not significant ($p > 0.05$), as the removal of paroxetine in the control with paroxetine (PRX Ct, 30%) was the same as presented in the abiotic control (PRX Cte). Paroxetine was not detected in the initial estuarine water.

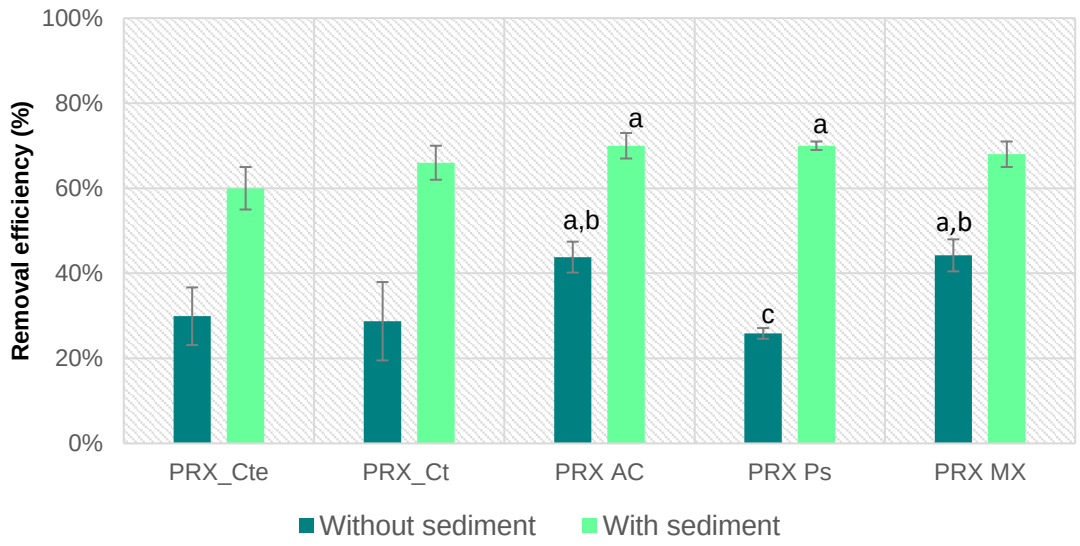

Figure 40 - Removal efficiency of paroxetine in the microcosms with and without sediment, in each treatment, after two weeks of experiment. Removal for microcosms with sediment were calculated taking into consideration the paroxetine detected in the sediments and water samples. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. a – significant differences among the sterile control (abiotic) and biotic treatments ($p < 0.05$). b - significant differences among the control (PRX Ct) and bioaugmented treatments ($p < 0.05$); c – significant differences among bioaugmented treatments. PRX Cte - sterile control; PRX Ct – control with paroxetine; PRX Ac – Bioaugmented with *Acinetobacter* DPS 5; PRX Ps – Bioaugmented with *Pseudomonas* DPS 10; PRX Mx– Bioaugmented with bacterial consortium.

4.5 Discussion

Pharmaceuticals have been reported in different environments, being classified as emerging pollutants. Technologies have been developed with the aim of improving the removal of pharmaceuticals, but most of the studies are focusing their efforts on the wastewater treatment plants. Even though wastewater treatment plants are one of the main inputs of pharmaceuticals in the environment, there is a lack of technologies suitable to be applied in natural environments, such as estuarine environments, rivers, among others. Bioremediation can be presented as a sustainable solution to remediate environments contaminated with pharmaceuticals. In this work, the aim was to evaluate the effects of bioaugmentation process, using native microorganisms, on the dynamics of estuarine natural community, as well as the potential of those microorganisms to degrade paroxetine in natural media.

The microorganisms used in this study were previous isolated from the Douro river estuary and their potential to degrade paroxetine in synthetic media was assessed (Fernandes et al., 2020). In this study, the bacterial consortium, that in a previous study displayed removal efficiency higher than 97% for paroxetine in synthetic culture media (Fernandes et al., 2020) was selected, as well two different strains, *Acinetobacter* sp. and *Pseudomonas* sp., belonging to the bacterial consortium.

The response of natural community present in the estuarine water and sediment to the different treatments was evaluated in terms of alpha diversity, beta diversity and taxonomic profile. Alpha diversity, evaluated through observed OTUs and Shannon diversity, showed a clear effect caused by the presence of paroxetine, as both metrics displayed a decrease in all treatments with the pharmaceutical (Figure 31). Beta diversity analysis performed by NMDS shaped two main groups, one made by initial sediment and Ct control samples and the other by samples treated with the pharmaceutical (Figure 32), bioaugmentation only presenting a slightly influence. These results were also confirmed by the taxonomic profile of the prokaryotic communities along the different sediment samples, where the effect of pharmaceutical was evident (Figure 33 and Figure 34). Statistical test (ANOSIM) confirmed the results obtained by community dynamics analysis, in which the most important factor on the shaping of the community structure was the pharmaceutical. Bioaugmentation was the factor that displayed lower statistical values (R^2) showing that bioaugmentation was not determinant for the changes observed in the natural community.

Experimental conditions did not influence the natural evolution of community structure (not treated). Indeed, looking at both alpha and beta diversity, in the end of the 2 weeks of the experiment, communities from microcosms without

pharmaceutical/pharmaceutical bioaugmentation were clearly different from those that were exposed to the pharmaceutical and/or bioaugmentation. Alpha diversity from initial sediment and microcosms assembled with both water and sediment (the sterile control of the experiment), representing the natural community subjected to laboratory conditions, displayed higher observed OTUs and diversity. This result highlights, on the other hand, that the effects of treatments in the microbial communities after two weeks, strongly changed in diversity and structure. Microbial communities are sensitive to environmental changes (Sun et al., 2012a; Zoppini et al., 2020), being capable to respond and adapt to stressful conditions (Fernandes et al., 2015). However, the presence of contaminants or other exogenous factors can lead to the disruption of the ecosystem, in which the natural community is not capable to respond.

The presence of paroxetine significantly affected the natural community from estuarine sediment, being the most important structuring factor. Effects of pharmaceuticals on microbial community structure, dynamics and diversity have been reported in different studies (Fernandes et al., 2015; Jechalke et al., 2014). Harrabi et al., 2019 studied the potential of microbial community from an estuarine sediment (same estuary selected in the present study) to biodegrade oxytetracycline and enrofloxacin, and they also evaluated the effects of both pharmaceuticals in the community structure using next-generation sequencing of the 16S rRNA gene amplicon. Both pharmaceuticals displayed effects on the community structure of the sediments, in which lower richness and diversity were observed in the cultures doped with target pharmaceuticals (Harrabi et al., 2019). In other study, it was evaluated the effects of vancomycin on river sediments bacterial community, using the DGGE approach being observed shifts in the community exposed to the pharmaceutical (Laverman et al., 2015).

Another factor responsible that was investigated regarding the shaping of the community structure was the bioaugmentation. In this study, the effects of bioaugmentation in the natural community was a crucial and important feature, as the aim is to reduce the impacts in the contaminated site, leading to their sustainable restauration. The effects of the bioaugmentation process on the natural community were not significant, displaying low statistical value in the ANOSIM test (Table 6). Fernandez et al. (2019) studied the response of microbial community from a tannery effluent to the bioaugmentation with a bacterial consortium(Fernandez et al., 2019).They observed that the addition of the bacterial inoculum did not display significant changes in the community structure, beyond the effects caused by the addition of the inoculum (Fernandez et al., 2019). A study conducted by Papadopoulou et al. (2018) showed that the bioaugmentation process, to degrade the

fungicide thiabendazole, did not significantly affect the alfa and beta diversity of bacterial community in the soil samples, which were collected in a field site that commonly receives wastewaters that contains thiabendazole and other compounds (Papadopoulou et al., 2018). In fact, as the bioaugmentation process is based on the addition of microorganisms, it is expected shifts on receiving community, but without causing the disruption of the affected environment. Thus, the most desirable outcome is to attain an effective removal of the pollutant by the microbial inoculum, without a significant and long term effects on the microbial community (Fernandez et al., 2019; Papadopoulou et al., 2018). There are other studies reporting different features regarding the effects of bioaugmentation on the community. Żur et al. (2020) studied the impact on the activity and functional capacity of the microorganisms from an activated sludge to the successive bioaugmentation of the activated sludge with the defined bacterial consortium (Żur et al., 2020). The authors observed that the addition of bacterial consortium did not significantly affect the biomass and metabolic activity of the microorganisms from activated sludge. Also, they reported that bioaugmentation strategy used in their study demonstrated to be promising to degrade poorly biodegradable pharmaceuticals, for example diclofenac and naproxen (Żur et al., 2020). In this study, no significant effect related with the bioaugmentation process was observed, agreeing with the reported data.

Taxonomic profile unveiled the composition of the microbial community in each treatment. The phyla Proteobacteria and Bacteroidetes were the most dominant in all treatments (Figure 33). The phylum Planctomycetes was also abundant in the initial sediment and control mimicking the natural conditions and, the phylum Euryarchaeota (belonging to the Archaea kingdom) was one of the most abundant in all treatments with pharmaceutical or both pharmaceutical and bioaugmentation. Phyla Proteobacteria, Bacteroidetes and Planctomycetes were previously found in the natural constitution of estuarine sediments. In a study conducted by Sheng et al. (2016) , using Illumina MiSeq targeting the V4 region of the bacterial 16S rRNA gene, Proteobacteria was found to be one of the most abundant phyla in an estuarine sediment from Poyang Lake (Sheng et al., 2016). In addition, the phyla Bacteroidetes and Planctomycetes were also a major component in the sediment community, where the presence of other phyla such as Acidobacteria, Firmicutes, Chloroflexi, Gemmatimonadetes, Actinobacteria, Nitrospirae, and Verrucomicrobia was also observed. Nair et al. (2017) performed a diversity analysis of marine sediment from Arabian Sea through metagenomics analysis, in which more than 60% was classified as Proteobacteria and 18% were classified to the Bacteroidetes phylum (Nair et al., 2017). In addition, the presence of Actinobacteria, Firmicutes and Chloroflexi

phyla were also observed, being in accordance with the results obtained in this study (Nair et al., 2017). In a study targeting the biodegradation of two antibiotics, Proteobacteria and Bacteroidetes were reported as the most abundant phyla in estuarine sediments collected in the Douro Estuary, close to the location from the sediments collected to conduct the present study (Harrabi et al., 2019).

At genus level (Figure 34), taxonomic profile showed that in the treatments with paroxetine, the genera *Methanococcoides* and *Methanolobus,* and Methylophagaceae family were the most abundant. *Methanococcoides* and *Methanolobus* are two genera belonging to the Archea domain (Euryarchaeota phylum), both of which are obligate methylotrophs (Munson et al., 1997). Archaea, including methanogenic Euryarchaeota, were reported by Haller et al. (2011) in contaminated sediments with high organic matter content (Haller et al., 2011). Yang et al. (2018) studied the anaerobic degradation of sulfamethoxazole in mangrove sediments and effects of sucrose and electron acceptors in the degradation efficiency, in which archaeal genera were identified in anaerobic SMX degradation microbial community for all methanogenic, sulfate-reducing and nitrate-reducing conditions (Yang et al., 2018a). The most abundant taxa were the Methylophagaceae family. These microorganisms are part of the Proteobacteria phylum, within the Gammaproteobacteria class. Very few reports about Methylophagaceae family were found. A study conducted by Bendia et al. (2018) unveiled the microbial community structure of sediment samples collected in fumaroles and glaciers at the geothermally active sites of Fumarole Bay and Whalers Bay, in Antarctica, in which Methylophagaceae was found in all sediment samples (Bendia et al., 2018).The three taxa described as the most abundant in this study are typically found in extreme environments (Bendia et al., 2018) or in environments in which methanogenesis is occurring (Singh et al., 2005; Ticak et al., 2014). The methanogenic archaea, a group of strictly anaerobic Euryarchaeota, can play an important role in the global carbon cycle, since its metabolism is restricted to the formation of methane from CO_2 and H_2, methanol, methylamines and/or acetate (Garcia et al., 2000; Thauer et al., 2008). In the present study, the microcosms were kept in static conditions in enclosed schott flasks, creating different biochemical environments throughout the experiment that can be more favorable for such taxa. These static conditions were chosen as they proved to be more favorable than agitated condition for the biodegradation of paroxetine (Duarte et al., 2019; Fernandes et al., 2020). At last, clam analysis also pointed Methylophagaceae family and the genera *Methanococcoides* and *Methanolobus* as specialists in the microcosms doped with paroxetine, indicating that these taxa may be responding to the presence of paroxetine. None of the bioaugmented taxa was found in

high abundance. Some of the bacterial strains were recovered from the corresponding treatment (Tables 7 and 8) and were detected by the local blast analysis (culture independent methods), however, they did not present a higher abundance in the bioaugmented treatments.

Potential of bioaugmentation process in natural media (estuarine water and sediment) was evaluated by measuring the removal efficiency of paroxetine. The consortium used in this study was obtained previously through a enrichment process with estuarine sediment sample, and was tested in synthetic culture media (mineral-salts medium) in co-metabolism with sodium acetate, presenting removal efficiencies higher than 97% (Duarte et al., 2019; Fernandes et al., 2020). Two strains from the consortium, *Acinetobacter* sp. and *Pseudomonas* sp., were selected to be tested alone as both showed potential to remove paroxetine in a previous study (chapter 3). Two different bioaugmentation approaches were tested to evaluate the best solution for the bioremediation process: by using a consortium or a single degrading strain. Different authors have been testing biodegradation of pharmaceuticals, using consortium or the single strain, both presenting promising results (Alexandrino et al., 2017; Pan et al., 2017; Zhang et al., 2013). To a better understanding regarding the main mechanisms of removal, microcosms were assembled as described in Figure 30. Microcosms with and without sediment were assembled to evaluate sorption processes as well as to evaluate the performance of the bacterial inoculum in both matrices. Sterile microcosms doped with paroxetine were assembled to evaluate abiotic degradation and control doped with paroxetine was assembled to evaluate the natural attenuation by the native community.

In the microcosms without sediment (Figure 40), microcosms inoculated with the strain *Acinetobacter* and with the consortium displayed significant removal efficiency compared with sterile control and control with paroxetine, showing removal efficiencies of 44%. However, most of the observed removal was due to abiotic degradation (30%). No significant natural attenuation was observed. Regarding the microcosms with sediment (Figure 40), removal efficiency of the bioaugmented treatments was around 70%, in which 60% of degradation was due to abiotic process. Paroxetine undergoes into abiotic degradation by photolysis (Kwon & Armbrust, 2004), being (3S,4R)-4-(4-fluorophenyl)-3-(hydroxymethyl) piperidine the main environmental metabolite (Kwon & Armbrust, 2004; Šakić et al., 2013). In synthetic culture media (Fernandes et al., 2020), the consortium used in this study was able to remove 97% of paroxetine, in static conditions. But in the present study, gathering all the results, it was considered that the bioaugmentation was not effective in promoting paroxetine removal. The lack of efficiency in this study can be due to different

factors such as amount of bacterial inoculum added to the microcosms, prokaryotic community response and adaptation, short time of the biodegradation experiment and a sort of environmental factors (availability of nutrients, organic matter and/or presence of other contaminants).

Adaptation of microbial community can highly influence the removal efficiency. In a previous study, Fernandes et al. (2015) showed that time of experiment significantly affected the bacterial structure of a constructed wetlands microcosms exposed to tetracycline or enrofloxacin (Fernandes et al., 2015). In addition, a crucial step in the bioaugmentation process is the adaptation and survival of the bioaugmented strains in the affected site (Vuković et al., 2019). So, in a short experiment, the bioaugmented strains may not had enough time to stablish and adapt properly, explaining the weakening performance.

A proper amount of bacterial inoculum is an important feature in the bioaugmentation process. The lack of efficiency observed in this study can be related with the insufficient bacterial inoculum used for the bioaugmentation process. A proper amount of bacterial inoculum should be added, as the bacterial inoculum should be able to compete with the autochthonous microbial community (Ozbayram et al., 2017). In the present study, it was used the same amount of bacterial inoculum used in the biodegradation experiments with synthetic media described in chapter 3 (optical density of 0.1). In addition, in the natural conditions simulated in the present study, the amount of bacterial inoculum that was added to the system may not be enough to promote paroxetine degradation, due to environmental constrains (different physical and chemical conditions and/or presence of other contaminants) and/or lack of competitive capacity to deal with natural microbial community.

Regarding the environmental factors, organic matter present in sediments can be crucial. In fact, the organic matter plays an important role in the bioavailability of contaminants and can have a negative effect on survival of inoculated strains and their ability to degrade the pollutant (Mrozik & Piotrowska-Seget, 2010). In a previous study, paroxetine showed high adsorption to cells and/or particles (Duarte et al., 2019). This propriety of paroxetine can compromise the capability of the bacterial inoculum to degraded it. Sorption of paroxetine onto sediments or biosolids was reported by several authors (Chari & Halden, 2012; Kwon & Armbrust, 2008; Radjenović et al., 2009; Wu et al., 2009). Paroxetine displays different K_{ow} values depending on the pH of the media (log K_{ow} = 1.35 for pH =7 and log K_{ow} = 3.95 for pH > 8) (Brown et al., 2015; Cunningham et al., 2004). Thus, depending on the pH and K_{ow} values, paroxetine can adsorb into sediments or particles that contains organic carbon as the cationic form of this compound is favoured in such conditions. The presence of other pollutants in the environment may also had an

influence on the removal efficiency. In natural environments, a complex mixture of other pollutants can result in synergistic effects, which can be toxic for the microbial community (Calisto & Esteves, 2009; Evgenidou et al., 2015). In addition, in this complex mixture, the presence of pollutants or compounds, more easily biodegradable, can affect the efficiency of bioaugmentation process for the target pollutant. In fact, lower substrate availability can induce microorganisms to use poorly degradable compounds as carbon source (Verlicchi et al., 2012). Other environmental factors such as temperature, salinity and pH can influence the bioaugmentation process, this should be addressed in future studies.

Bioaugmentation efficiency can also be inhibited or diminished by insufficient nutrient availability, more specifically, phosphorus and nitrogen (Mrozik & Piotrowska-Seget, 2010; Roy et al., 2018; Smith et al., 2015). For the microcosms assembled with sediment (Figure 39), trace levels of nitrite, nitrate and phosphate were detected in all bioaugmented systems, in which significant increasing of ammonium was observed for the treatments bioaugmented with the strain *Pseudomonas* and the consortium. The high levels of ammonium can be explained by the ammonification process, in which heterotroph microorganisms can enzymatically degrade different forms of organic nitrogen into $N–NH_4^+$ (Guillen-Jimenez et al., 2000).

In the microcosms without sediment, the levels were slightly higher compared with the microcosms with sediment, except for phosphate levels, in which all nutrients significantly decreased compared with the control. Overall, the nutrient levels in the bioaugmented systems were not enough to stimulate the community and enable the degradation of the pharmaceutical. In fact, the absence of available nutrients in the impacted environment can compromise the bioremediation process as the scarcity of suitable nutrients can inhibit or diminish the performance of microbial community. So, in the future, more tests should be performed in order to optimize a suitable nutrient formulation to complement the bioaugmentation process.

4.6 Conclusions

In this study, bioaugmentation in natural media using natural estuarine water and sediment was attempted. Results showed that bioaugmentation did not present a clear effect on the community structure. In fact, the addition of pharmaceutical displayed shifts in community dynamics, as showed by different analysis (alfa and beta diversity, ANOSIM, and taxonomic profile). Low removal efficiency of paroxetine was observed as a limitation on the nutrient content in natural media may had led to a lower stimulation of the natural and bioaugmented community. In the future, bioaugmentation combined with biostimulation

should be considered, as well as the monitoring of different factors that can lead to environmental constrains, as the presence of other pollutants and organic matter content.

Chapter 5

General Discussion and Conclusions

5.1 General discussion

In this work, the development of a bioremediation solution to remove paroxetine and bezafibrate was assessed. From the enriched cultures to experiments in natural media, this work showed the potential of native microorganisms isolated in estuarine sediments, to remove the selected pharmaceuticals. Still, this work also showed that there are several steps that should be optimized and considered in future works and should be taken into consideration in the development of a bioremediation technology.

This work started with five enriched cultures, previously obtained by Duarte and collaborators (Duarte et al., 2019).To contextualize, these enriched cultures were obtained exposing estuarine sediment and activated sludge as source of inocula to paroxetine and bezafibrate, and incubated in static and agitated conditions. At the end of the mentioned experiment, eight enriched cultures were obtained (four in static and four in agitation), with capability to degrade the selected pharmaceuticals. Since the enriched cultures in static conditions presented the best degrading performances, they were selected for this work. One culture in agitation, from activated sludge inoculum, was also selected, as more representative of the activated sludge system, that is constantly in agitation.

The enrichment process is an important step for the selection of microorganisms with capability to degrade a specific compound (Gaskin & Bentham, 2005). A direct isolation from the source of inoculum could be performed, however, with this approach, several microorganisms can be selected and isolated but without the ability to degrade the compound. The enrichment process imposes a selective pressure on the microbial community of the inoculum, selecting the microorganisms with the capability to degrade the compound or to survive and grow in its presence. The limitation of the enrichment process is related with the fact that only a small percentage of the microorganisms can be culturable (Vartoukian et al., 2010). However, this limitation is also valid for the direct isolation from the source of inoculum. Therefore, it is possible that after the enrichment, some crucial microorganisms can be lost during the isolation process. Despite this limitation, the isolation of the microorganisms that are in the enriched cultures is vital for the development of the bioremediation technology, as for this purpose, only the culturable bacteria can be used to produce bacterial biomass for future application for in situ bioremediation of contaminated sites. Thus, in the chapter 2, the isolation of the culturable bacteria from the five selected enriched cultures was performed. Two different culture media were used, the MM agar supplemented with the target pharmaceutical, and the PCA. The MM agar was tested since it was the same medium used in the enrichment process. On the other hand, PCA is a non-selective and richer medium, that was selected in the attempt to recover different isolates

with less affinity to the MM medium. In this step, other microorganisms can also be lost, since the two selected media for the isolation process can not be suitable for all the culturable bacteria present in the enriched culture.

After the enrichment process and isolation in two different media, in total forty-eight bacterial isolates were retrieved from the five enriched cultures. The next step was to reassemble these bacterial strains into five consortia, according to the enriched culture from which they were derived. This is a very important step, in which the capability of the reassembled consortia to degrade the pharmaceutical was compared with the degradation potential of the respective enriched culture. By doing so, it is possible to infer if some of the degrading bacteria was lost during the isolation process. In this study, it was shown that both consortia derived from estuarine sediment (one pre-enriched with paroxetine and other pre-enriched with bezafibrate) maintained the removal capability to degrade the pharmaceuticals, comparing with the removal efficiencies obtained for the respective enriched cultures, indicating that the key bacterial strains involved in the degradation were recovered (Figure 9, chapter 2). The same was observed for the consortia derived from activated sludge, pre enriched with bezafibrate. For the other two consortia derived from activated sludge, one in static conditions and pre-enriched with paroxetine and the other in agitated conditions and pre-enriched with bezafibrate, the removal efficiency decreased comparing with the respective enriched cultures, suggesting that some of the key bacteria may have not been successfully isolated (unculturable bacteria). In addition, the decreasing on the removal can be also related with acclimation needs, as these cultures may need more time to acquire the optimal conformation to achieve the highest performance. Thus, three of the five enriched cultures were able to remove more than 97% of paroxetine or bezafibrate, showing that the enrichment process and the isolation procedure allowed the selection and isolation of bacterial strains with potential to degrade the target compounds.

Different incubation conditions, static and agitation, could also have influenced the enrichment process and selection of the degrading strains. In fact, with different incubation conditions, different biochemical environments are imposed leading to the enrichment of different degrading bacteria. In the case of static conditions, an oxygen gradient is created allowing the growth of bacteria with different respiratory requirements. As the case of agitated conditions, the oxygen is gradually dissolved throughout the medium stablishing similar conditions in the microcosms. In this study, the consortia in static conditions showed higher performances comparing with the ones in agitated conditions. In addition, the taxonomic identification of the bacterial strains from each bacterial consortium showed that different bacterial genera were selected for different enrichment conditions (Figures 4 and

5, chapter 2) The genus *Pseudomonas* was recovered from all enriched cultures, despite the incubation conditions or the type of pharmaceutical that was used for the enrichment. Also, this genus was the most abundant in all enriched cultures. However, the genus *Acinetobacte*r was only isolated in enriched cultures obtained in static conditions. Moreover, *Microbacterium* was only detected in the enriched cultures derived from estuarine sediment, also in static conditions. The genus *Hydrogenophaga* was detected in both cultures obtained from activated sludge inoculum in static conditions. The genera *Shewanella* and *Leadbetterella* were detected only in cultures exposed to paroxetine. Most of the bacterial genera that were retrieved from the five enriched cultures were reported to be involved in the degradation of other pharmaceuticals (Jiang et al., 2014; Mao et al., 2018; Wang et al., 2018; Woźniak-Karczewska et al., 2018) however, to our best knowledge, none of these strains was reported to be involved in the degradation of paroxetine or bezafibrate.

Thus, the enrichment process allowed the selection of potential degrading strains for the removal of paroxetine and bezafibrate, an important step for the development of the bioremediation technology. In addition, potential new species were selected in all enriched cultures (Figures 6-8). The next step was the optimization of the bacterial consortia, by accessing the potential of each bacterial strain for the removal of the respective pharmaceutical. During the enrichment process, bacterial strains with capability to degrade the pharmaceutical or bacterial strains that can adapt and survive in its presence can be selected. So, it is important to evaluate which are the ones with the capability to degrade the compound. For the optimization experiments, described in chapter 3, both consortia derived from estuarine sediment inocula, paroxetine degrading consortium (DPS consortium) and bezafibrate degrading consortium (DBS consortium), were selected. These two consortia were selected as both displayed high removal efficiency in the experiments conducted in chapter 2 (Figure 9). In addition, the main goal was to develop a technology suitable to be applied in natural environments.

The consortia obtained for the activated sludge inocula should be addressed in future woks, as it is also important to improve the removal efficiency of pharmaceuticals in the wastewater treatment facilities. WWTPs are the main input of pharmaceuticals in the environment (Comber et al., 2018). These facilities were not designed to remove pharmaceuticals, so most of them are released to the environment. An improvement on the removal efficiency of WWTPs could prevent the entrance of different pharmaceuticals in the environment. There are several studies addressing the removal of pharmaceuticals in WWTPs (Afonso-Olivares et al., 2017; Comber et al., 2018) and exploring new technologies (Changotra et al., 2019; Kårelid et al., 2017), however, there is a lack of studies addressing

the removal of pharmaceuticals in natural environments. Thus, for all these reasons, the consortia from estuarine sediment were selected.

The optimization of the paroxetine and bezafibrate degrading consortia was performed and described in chapter 3. The optimization of the bacterial consortium is an important feature for the development of a biotechnological tool. The use of a large number of different bacterial strains can increase the complexity and the costs regarding the production of the bioremediation formula. In addition, bacterial strains with a stable bacterial growth in small experiments, can represent a challenge when the production of a large amount of biomass is attempted. Therefore, the use of a reduced number of strains in a bacterial consortium is an important feature for further experiments. In this regard, the potential of each bacterial strain was evaluated for the development of an optimized consortia. The optimization processes started with the paroxetine degrading consortium, in which the potential of each bacterial strain was evaluated. The strains *Pseudomonas* DPS 10, *Pseudomonas* DPS 1, Chitinophagaceae family DPS 4 and *Leadbetterella* DPS 8 were the best degrading strains with removal efficiencies between 81% – 99% and defluorination values between 64% – 77%. As described in more detail in chapter 2 and chapter 3, the bacterial genera *Pseudomonas* and *Leadbetterella* and the family Chitinophagaceae were found to be related/involved with the degradation of other pharmaceuticals (Kostanjevecki et al., 2019; Rutere et al., 2020; Yang et al., 2020). In addition, *Leadbetterella* DPS 8 and the Chitinophagaceae family DPS 4 are among the potential new species isolated (chapter 2), with capability to degrade paroxetine. Furthermore, DPS consortium was also assembled to compare the degradation performed by the bacterial strains and the consortium in the same conditions. This consortium was able to remove up to 85% of paroxetine, in which almost 80% was defluorinated. A decreasing on the performance of DPS consortium was observed comparing with the one described in chapter 2.

The following step was the assembling of bacterial consortium (OPDPS) with the selected strains: Chitinophagaceae family DPS 4, *Leadbetterella* DPS 8 and *Pseudomonas* DPS 10. OPDPS consortium was able to defluorinate almost 50% of the molecule, a slightly lower value comparing with the consortium assembled with the 10 bacterial strains in the same experiment. However, the DPS consortium displayed a significant decreasing on the performance, being only able to defluorinate 55% of the molecule. Cryopreservation and reactivation processes were the most likely factors related with the abrupt decreasing observed among experiments. Still, other factors that could have affected the removal efficiency were evaluated, such as the bacterial growth of each strain on the selected carbon source (sodium acetate), time of cultivation of each bacterial strain before inoculation

(related with the reactivation process) and the use of different starting OD for the beginning of the experiments.

Starting with the bacteria growth, in the experiments with each bacterial strain, the lack of defluorination observed for some strains was related with the poor bacterial growth. In addition, two of the strains selected for the OPDPS, Chitinophagaceae family DPS 4 and *Leadbetterella* DPS 8, displayed erratic bacterial growth among experiments. Consequently, the bacterial growth for each bacterial strain in the presence of acetate but without the presence of paroxetine was evaluated (Figure 17). Then, the strains with lower bacterial growth were selected for the experiments with different carbon sources. In fact, it was observed an improvement on the bacterial growth for *Bosea* DPS 2, *Leadbetterella* DPS 8 and Chitinophagaceae family DPS 4 in the presence of yeast extract (Figure 18).

In the case of bezafibrate, the experiments for the optimization of DBS bacterial consortium started by testing the bacterial growth of each strain in the presence of sodium acetate, followed by the experiment with different carbon sources. These steps were performed before the biodegradation experiments to avoid growing issues related with second carbon source. The strains *Dyadobacter* DBS3*, Leucobacter* DBS 5 and *Microbacterium oxydans* DBS 10 displayed higher bacterial growth in yeast extract, being this carbon source selected for further experiments. Moving for the biodegradation experiments evaluating the potential of each bacterial strain from DBS consortium to degrade bezafibrate, *Dyadobacter* DBS 3*, Leucobacter* DBS 5, *Microbacterium oxydans* DBS 10 and *Ochrobactrum rhizosphaerae* DBS 4 were the best degrading strains. Once more, two potential new species, *Dyadobacter* DBS3 and *Leucobacter* DBS 5 showed potential to degrade, in this case, bezafibrate. Regarding the DBS consortium, only 65% of bezafibrate was removed, showing a significant decrease comparing with the removal obtained in chapter 2.

One of the hypotheses to explain the decreasing on the removal efficiency and defluorination was related with the OD that was used in the experiments from chapter 2 and chapter 3. In the chapter 2, the biodegradation experiments were assembled with an OD of 0.5, whereas in the experiments from chapter 3, the experiments were assembled starting with an OD of 0.1. Thus, a small experiment addressing this hypothesis was performed for both DPS and DBS consortia, however, no significant differences were observed between the experiments with an OD of 0.1 and 0.5. In addition, a new decreasing on the performance was observed for both consortia. Thus, the OD was not related with the lack of removal efficiency. Thus, other hypothesis was considered, related with the reactivation of the bacterial strains before the biodegradations experiments. First, a small growth

experiment was conducted with the bacterial strain Chitinophagaceae family DPS 4, in which was explored different cultivation periods before inoculation in MM media. For that, bacterial preserved at -80 °C cultivated in PCA plate for one week and bacterial strain preserved at -80 °C and cultured in PCA media for 2 weeks, were inoculated in MM media with sodium acetate. Results showed that bacterial strain cultivated in PCA plate only for a one week required more time to growth in the MM media comparing with the one that was cultivated in PCA plate for 2 weeks and a half. So, considering this results, new experiment was conducted using the DPS consortium testing a different cultivation period (two and half weeks instead of one week), after retrieving from -80°C. DPS consortium reached 100% of defluorination, as reported in the chapter 2, showing that reactivation of bacterial strains had an influence on the removal efficiency across the experiments.

At the end, the results strongly indicated that cryopreservation and reactivation processes may have influenced the removal efficiency observed for both consortia. Optimization of cryopreservation and reactivation processes should be addressed in future experiments, testing the possibility of using different cryoprotectants, evaluate the effect of using different cultivation times before the experiment and the possibility of using an acclimatation period before the biodegradation experiment, in which the bacterial strain or the consortium is exposed to the pharmaceutical and fed with a second carbon source only for adaptation of the strains to the new environment. In the chapter 3, it was not possible to reach a final optimized consortium. More biodegradation experiments are needed in the future to test different combinations among the ten bacterial strains. There is also the hypothesis to be tested that the ten bacterial strains are needed to reach the maximum performance.

This study started with experiments in synthetic media, to reach the ideal conditions to achieve the maximum performance. Afterwards, the experiments in natural media (chapter 4) were conducted. Application of bioremediation on natural media encompasses more complexity, in which several factors can interfere with the removal of the pollutant. Organic matter content, salinity gradients, the presence of a cocktail of different organic and inorganic pollutants and competition with the natural community are examples of different constrains that can be found in these environments. The previous experiments were conducted in a poor culture media spiked with the pharmaceutical and a second carbon source, pressing the bacterial strains or the consortium to use the pharmaceutical as substrate. In natural media, the bioaugmented strains could use other pollutants as substrate or use other compounds in the matrix, that could be more easily degraded by them. In addition, despite the use of autochthonous microorganisms, they have to compete

with the natural community for nutrients and carbon source and adapt again to the new environmental conditions.

So, in the experiments in natural media described in chapter 4, it was explored the bioaugmentation process for the removal of paroxetine. Moreover, this study aimed to evaluate the effects of bioaugmentation process in the microbial community dynamics using Illumina sequencing of 16S rRNA gene. Assessment of possible impacts caused by bioaugmentation process in the natural community dynamics should be addressed for a full understanding of the ecological effects of the bioremediation process.

For experiments in natural media, the DPS consortium and two bacterial strains, *Pseudomonas* DPS 10 (one of the best degrading strains) and *Acinetobacter* DPS 5 were selected. The strains Chitinophagaceae family DPS 4 and *Leadbetterella* DPS 8 were not selected to be used in a more complex media, since earlier they presented erratic bacterial growth. *Acinetobacter* DPS 5 demonstrated higher bacterial growth and removal efficiencies for paroxetine (higher than 65%). In addition, the genus *Acinetobacter* has been reported to be involved in the degradation of other pharmaceuticals, as previously mentioned (Wang et al., 2018; Zhang et al., 2012).

The selected strains and the bacterial consortium were tested in experiments using estuarine water and sediment (representing the natural media), collected in the same geographic area as the sediment used for the enrichment process. Microcosms were assembled, in which half contained only estuarine water, and the other half estuarine water and sediment. This procedure was performed to better understand the dynamics on the removal of paroxetine. The microcosms were spiked with paroxetine (1 mg L^{-1}) and bioaugmented with each bacterial strain or the consortium, with an OD of 0.1. This OD was selected since most of the experiments conducted in the chapter 3 were performed with this OD. In addition, previously, for the proportion of paroxetine added to the system (regarding the input of carbon source) this amount of bacterial inocula proved to be efficient to remove it. Furthermore, a lower amount of bacterial inoculum was chosen since an excessive amount of bacterial inoculum could lead to a complete dominance of the native community by the bioaugmented inocula and a sudden consumption of the available nutrients, disrupting the microcosm community.

The effect of paroxetine on the microbial dynamics was also evaluated, in which, the microcosms with the natural community were exposed to paroxetine, without the presence of the bioaugmented strains. With this control, it was also possible to observe the responses of the natural community to the pharmaceutical and measure the natural attenuation that occurred throughout the experiment. The effects on the natural community due to the

experimental conditions was also addressed by assembling a microcosm only with estuarine water and sediment. Illumina sequencing of 16S rRNA gene was only performed in the microcosms assembled with estuarine water and sediment, as more representative of the natural conditions. In this case, only the sediment samples from each treatment were evaluated.

Experimental conditions did not significantly affect the natural community, as confirmed by the ANOSIM test. In fact, both alpha diversity indicators showed that the community exposed to the laboratorial conditions (Ct) displayed similar OTUs and Shannon diversity comparing with the community displayed by the initial sediment (IS) (Figure 31). In addition, in the NMDS analysis, the same pattern was observed, in which this natural control (Ct) was grouped with the initial sediment (IS) (Figure 32). With this result, the changes observed on the community structure can be correlated with the bioaugmentation process and the addition of the pharmaceutical. Bioaugmentation process did not significantly affect the community structure, as showed by the ANOSIM test. No significant effects were observed on alpha and beta diversity, comparing with the control (control with paroxetine, PRX Ct). The low impact of the bioaugmentation process is a very positive outcome, since the main objective with the bioaugmentation process is to attain an effective removal of the pollutant by the bacterial inoculum, without significant and long term effects on the microbial community (Fernandez et al., 2019; Papadopoulou et al., 2018). However, shifts on the community were expected, as the natural community has to compete with the added bacteria for nutrients and carbon source. The low impact of the bioaugmentation process can also be related with the low amount of bacterial inocula that was added to the microcosms, this amount might not have been enough to induce a significant response of the community. On the other hand, paroxetine significantly affected the community. In fact, all microcosms spiked with paroxetine showed a decrease on the alpha diversity, for both Observed OTU and Shannon diversity. In addition, in the NMDS analysis, all microcosms doped with paroxetine were grouped together. Changes on the community structure induced by the presence of pharmaceuticals were already reported by other authors (Alexandrino et al., 2017; Davids et al., 2017; Fernandes et al., 2015; Hu et al., 2018; Laverman et al., 2015).

Paroxetine was not efficiently removed from the natural media. In fact, low removal efficiency was observed in the microcosms with and without sediment. In the microcosms with sediment, higher removal was observed comparing with the microcosms without sediment. Moreover, paroxetine was detected in sediment samples. The absorption of paroxetine into sediments (Kwon & Armbrust, 2008) or cells (Duarte et al., 2019) was

reported before. The low K_{ow} (1.35 for pH =7 (Cunningham et al., 2004; Kwon & Armbrust, 2008) associated with paroxetine pK_a (10.32), can favour the cationic form of paroxetine in environmental condition, explaining the sorption capability of paroxetine (Kwon & Armbrust, 2008). Bioaugmentation process can be inhibited or constrained by different factors such as the presence of a high pollutant concentration, pH, organic matter content, unbalance nutrient levels, among others (Mrozik & Piotrowska-Seget, 2010; Roy et al., 2018; Smith et al., 2015). In addition, the amount of bacterial inoculum can also affect the bioaugmentation efficiency, as a proper amount of bacterial inoculum must be added to allow the bioaugmented community to compete with the natural microbial community (Ozbayram et al., 2017). In this study, nutrient levels significantly decreased in the microcosms with and without sediment, including in the control with the natural community pointing that nutrient levels were not enough to stimulate the bioaugmented community, affecting the removal efficiency. Also, as mentioned before, a low bacterial inoculum was used (OD 0.1) which may not be enough to promote paroxetine degradation. Moreover, the bioaugmented bacteria may experience difficulties to adapt and compete with the natural community. In addition, the presence of other pollutants can represent a hindrance for the bioaugmentation performance. With the presence of other carbon sources, the bioaugmented bacteria can degrade other compounds presented in the media and leave the target compound practically unbothered. To be certain, a pollutant characterization before and after experiment should be performed to assess if the bioaugmented bacteria degraded other compounds. Thus, the natural media experiments encompass several environmental constrains that should be address, in order to improve the bioremediation process.

5.2 Conclusions

This work explored the potential of native microorganisms for the biodegradation of two pharmaceuticals, paroxetine and bezafibrate in synthetic and natural media.

Five bacterial consortia able to degrade paroxetine and bezafibrate were obtained. In addition, this work unveiled the potential of different bacterial strains for the degradation of paroxetine and bezafibrate, that may represent an important contribution for the development of new bioremediation tools to remove pharmaceuticals from contaminated environments. Moreover, this work revealed for the first time the taxonomic diversity associated with the biodegradation of paroxetine and bezafibrate, in which potential new species were isolated and presented potential to degrade the selected pharmaceuticals.

In the future, several steps should be optimized. As discussed before, cryopreservation of the bacterial strains can affect their metabolism and viability. In addition,

an improper reactivation can strongly affect the performance of the bacteria strains. Thus, in the future, experiments addressing these features are needed, since without a proper storage and reactivation, the efficiency can drastically decrease. Regarding the experiments in natural media, a combination of both bioaugmentation and biostimulation should be considered, to enhance and stimulate the biological activity of the bacterial inocula and the natural community, to fully assess autochthonous bioaugmentation potential. In addition, different environmental constrains, as the presence of other pollutants and organic matter content should be evaluated as they can interact, inhibit or decrease the effectiveness of the bioaugmentation process.

The work developed in this PhD project contributed with new insights on the development of bioremediation tools to remove pharmaceuticals from contaminated environments.

Chapter 6

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Appendix

Appendix I

Chapter 1

Table S 1 - Pharmaceuticals concentration detected in freshwater samples (expressed in ng L^{-1}).

Therapeutic Class	Pharmaceutical	Location	Concentration (ng L^{-1})	Reference
Antibiotics	Ciprofloxacin	Spain	34.6	Vazquez-Roig et al. (2012)
		Spain	740	Gracia-Lor et al. (2011)
		Spain	23	Gros et al. (2012)
		Spain	0.53 – 20.00	Osorio et al. (2016)
		Serbia	28.2	Petrović et al. (2014)
		Spain	34.6	Vazquez-Roig et al. (2010)
		Spain	17 - 540	Mijangos et al. (2018)
	Norfloxacin	Spain	37.2	Vazquez-Roig et al. (2012)
		Spain	54	Gracia-Lor et al. (2011)
		Spain	37.2	Vazquez-Roig et al. (2010)
		Spain	5 - 62	Mijangos et al. (2018)
	Ofloxacin	Spain	50.2	Vazquez-Roig et al. (2012)
		Spain	400	Gracia-Lor et al. (2011)
		Spain	20 - 33	Gros et al. (2012)
		Spain	0.07 – 109.50	Osorio et al. (2016)
		France	3.2	Vulliet & Cren-Olivé (2011)
		Spain	50.2	Vazquez-Roig et al. (2010)
	Sulfamethoxazole	Spain	15.6	Vazquez-Roig et al. (2012)
		Portugal	9.14 - 53.3	Madureira et al. (2010a)
		Spain	33	Gracia-Lor et al. (2011)

Antibiotics	Sulfamethoxazole	USA	66.7	Klosterhaus et al. (2013)
		Spain	16 - 79	Gros et al. (2012)
		UK	1.8	Petrie et al. (2016)
		Spain	0.16 – 41.51	Osorio et al. (2016)
		France	1.9	Vulliet & Cren-Olivé (2011)
		Spain	15.6	Vazquez-Roig et al. (2010)
		Spain	19 - 227	Mijangos et al. (2018)
		Mexico	76 - 722	Rivera-Jaimes et al. (2018)
	Trimethoprim	Spain	3.0	Vazquez-Roig et al. (2012)
		Portugal	3.89 to 15.7	Madureira et al. (2010a)
		Spain	151	Gracia-Lor et al. (2011)
		USA	4.1	Klosterhaus et al. (2013)
		Spain	5 - 9	Gros et al. (2012)
		UK	22	Petrie et al. (2016)
		Spain	0.49 – 150.43	Osorio et al. (2016)
		Serbia	8.1	Petrović et al. (2014)
		France	0.9	Vulliet & Cren-Olivé (2011)
		Spain	3.0	Vazquez-Roig et al. (2010)
		Spain	3 - 2046	Mijangos et al. (2018)
		Mexico	34 - 120	Rivera-Jaimes et al. (2018)
	Erythromycin	UK	132 - 1378	Kay et al. (2017)
		Spain	78	Gracia-Lor et al. (2011)

Antibiotics	Erythromycin	Spain	0.45 – 18.58	Osorio et al. (2016)
		Serbia	292	Petrović et al. (2014)
		Portugal	38.80	Pereira et al. (2017)
	Erythromycin – H_2O	USA	12.1	Klosterhaus et al. (2013)
	Clarithromycin	Spain	91	Gracia-Lor et al. (2011)
		USA	17.6	Klosterhaus et al. (2013)
		Spain	59	Gros et al. (2012)
		UK	43.5	Petrie et al. (2016)
		Spain	0.09 – 65.63	Osorio et al. (2016)
		Serbia	616	Petrović et al. (2014)
		Portugal	39.10	Pereira et al. (2017)
	Enrofloxacin	Spain	70	Gracia-Lor et al. (2011)
	Lincomycin	Spain	47	Gracia-Lor et al. (2011)
	Pefloxacin	Spain	64	Gracia-Lor et al. (2011)
	Roxithromycin	Spain	12	Gracia-Lor et al. (2011)
		France	4.9	Vulliet & Cren-Olivé (2011)
	Sarafloxacin	Spain	55	Gracia-Lor et al. (2011)
	Sulfamethizole	USA	15.6	Klosterhaus et al. (2013)
	Flumequine	Spain	20	Gracia-Lor et al. (2011)
	Azithromycin	Spain	5 - 41	Gros et al. (2012)
		Spain	2.26 – 33.22	Osorio et al. (2016)

Antibiotics	Azithromycin	Portugal	35.66	Pereira et al. (2017)
	Cefalexin	Spain	0.40 – 1.40	Osorio et al. (2016)
		Serbia	283	Petrović et al. (2014)
	Tetracycline	Spain	5.92 – 27.40	Osorio et al. (2016)
	Nalidixic Acid	Spain	14	Gracia-Lor et al. (2011)
	Oxolinic Acid	Spain	23	Gracia-Lor et al. (2011)
	Pipedimic acid	Spain	245	Gracia-Lor et al. (2011)
	Metronidazole	Spain	0.96 – 65.93	Osorio et al. (2016)
		France	0.3	Vulliet & Cren-Olivé (2011)
	Moxifloxacin	Spain	205	Gracia-Lor et al. (2011)
	Mefenamic acid	UK	9 - 97	Kay et al. (2017)
	Sulfadiazine	Spain	7 - 51	Mijangos et al. (2018)
Nonsteroidal anti-inflammatory drug (NSAIDs)	Diclofenac	Spain	16.9	Vazquez-Roig et al. (2012)
		France	1.36 – 33.2	Togola & Budzinski (2008)
		UK	76 - 2991	Kay et al. (2017)
		Spain	16.9	Vazquez-Roig et al. (2010)
		Spain	358	Gracia-Lor et al. (2011)
		Spain	18 - 52	Gros et al. (2012)
		UK	21.5	Petrie et al. (2016)
		Spain	26.63 - 280	Osorio et al. (2016)
		Serbia	324	Petrović et al. (2014)

Nonsteroidal anti-inflammatory drug (NSAIDs)	Diclofenac	France	5.4	Vulliet & Cren-Olivé (2011)
		Spain	22 - 650	Mijangos et al. (2018)
		Portugal	51.24	Pereira et al. (2017)
		Mexico	258 - 1398	Rivera-Jaimes et al. (2018)
	Ibuprofen	Spain	59.0	Vazquez-Roig et al. (2012)
		France	4.5	Togola & Budzinski (2008)
		UK	205 - 4838	Kay et al. (2017)
		Spain	2850	Gracia-Lor et al. (2011)
		USA	37.9	Klosterhaus et al. (2013)
		Spain	380	Gros et al. (2012)
		UK	27.5	Petrie et al. (2016)
		Spain	3.91 – 867.82	Osorio et al. (2016)
		Serbia	346	Petrović et al. (2014)
		France	5.5	Vulliet & Cren-Olivé (2011)
		Spain	59	Vazquez-Roig et al. (2010)
		Mexico	184 - 1106	Rivera-Jaimes et al. (2018)
	Ketoprofen	France	14.5	Togola & Budzinski (2008)
		Spain	70	Gracia-Lor et al. (2011)
		Spain	6.37 – 356.79	Osorio et al. (2016)
		Serbia	45	Petrović et al. (2014)
		France	3.4	Vulliet & Cren-Olivé (2011)
		Spain	4 - 57	Mijangos et al. (2018)

Nonsteroidal anti-inflammatory drug (NSAIDs)	Tenoxicam	Spain	0.02 – 1.59	Osorio et al. (2016)
	Naproxen	France	9.1	Togola & Budzinski (2008)
		Spain	285	Gracia-Lor et al. (2011)
		USA	8.2	Klosterhaus et al. (2013)
		Spain	7 - 156	Gros et al. (2012)
		UK	127	Petrie et al. (2016)
		Spain	12.21 – 289.47	Osorio et al. (2016)
		Serbia	74.2	Petrović et al. (2014)
		France	3.5	Vulliet & Cren-Olivé (2011)
		Mexico	732 - 4880	Rivera-Jaimes et al. (2018)
	Piroxicam	Spain	5 - 11	Gros et al. (2012)
		Spain	0.03 – 5.06	Osorio et al. (2016)
	Meloxicam	Spain	74	Gros et al. (2012)
		Spain	0.01 – 4.00	Osorio et al. (2016)
		Serbia	1.8	Petrović et al. (2014)
	Indomethacin	Spain	11	Gros et al. (2012)
		Spain	1.55 – 137.44	Osorio et al. (2016)
		Serbia	19.5	Petrović et al. (2014)
	Indomethacin	Mexico	19 - 362	Rivera-Jaimes et al. (2018)
	Phenazone	Spain	12	Gros et al. (2012)
		Spain	0.07 – 40.72	Osorio et al. (2016)
		Serbia	12.5	Petrović et al. (2014)

Antidepressants	Norfluoxetine	Spain	0.84 - 3.26	Osorio et al. (2016)
	Sertraline	Spain	1.06 – 144.87	Osorio et al. (2016)
		Portugal	23.30	Pereira et al. (2017)
	Paroxetine	Spain	40	Gros et al. (2012)
		Spain)	0.27 - 3.41	Osorio et al. (2016)
	Venlafaxine	Spain	575	Gracia-Lor et al. (2011)
		Spain	43	Gros et al. (2012)
		UK	31.1	Petrie et al. (2016)
		Spain	1.15 – 127.62	Osorio et al. (2016)
		Serbia	5.3	Petrović et al. (2014)
	Citalopram	Spain	9 - 11	Gros et al. (2012)
		Spain	0.08 – 31.83	Osorio et al. (2016)
		Portugal	52.97	Pereira et al. (2017)
	Trazadone	Spain	4	Gros et al. (2012)
		Spain	0.04 – 40.04	Osorio et al. (2016)
	Fluoxetine	Spain	0.59 – 17.28	Osorio et al. (2016)
Blood lipid lowering agents	Fenofibrate	Spain	21.4	Vazquez-Roig et al. (2010)
	Gemfibrozil	France	2.3	Togola & Budzinski (2008)
		USA	38.2	Klosterhaus et al. (2013)
		Spain	304	Gracia-Lor et al. (2011)

Blood lipid lowering agents	Gemfibrozil	Spain	9.80 – 302.67	Osorio et al. (2016)
		Spain	22 - 284	Gros et al. (2012)
		Portugal	10.34	Pereira et al. (2017)
		Mexico	9 - 368	Rivera-Jaimes et al. (2018)
	Pravastatine	France	1.6	Vulliet & Cren-Olivé (2011)
		Spain	0.53-10.81	Osorio et al. (2016)
	Bezafibrate	France	3.4	Vulliet & Cren-Olivé (2011)
		UK	42.1	Petrie et al. (2016)
		Spain	49	Gracia-Lor et al. (2011)
		Spain	0.82 – 55.64	Osorio et al. (2016)
		Spain	16	Gros et al. (2012)
		Serbia	18.18	Petrović et al. (2014)
		Spain	8 - 67	Mijangos et al. (2018)
		Portugal	15.52	Pereira et al. (2017)
		Mexico	286 - 2100	Rivera-Jaimes et al. (2018)
	Atorvastatin	UK	7	Petrie et al. (2016)
		Spain	42	Gracia-Lor et al. (2011)
		Spain	0.12 – 8.64	Osorio et al. (2016)
		Spain	2-3	Gros et al. (2012)
		Serbia	9.09	Petrović et al. (2014)
	Fluvastatin	Spain	0.06 – 4.19	Osorio et al. (2016)
	Fenofibric acid	France	0.6	Vulliet & Cren-Olivé (2011)

Table S 2 - Pharmaceuticals concentration detected in underground water samples (expressed in ng L^{-1}).

Therapeutic Class	Pharmaceutical	Location	Concentration (ng L^{-1})	Reference
Antibiotics	Azithromycin	Spain	30.7 – 1620	López-Serna et al. (2013)
	Clarithromycin	Spain	2.87 – 20.5	López-Serna et al. (2013)
	Chlortetracycline	Spain	34.2	López-Serna et al. (2013)
	Ciprofloxacin	Spain	51 – 443	López-Serna et al. (2013)
	Danofloxacin	Spain	58.7 - 543	López-Serna et al. (2013)
	Doxycycline	Spain	27.6 – 188	López-Serna et al. (2013)
	Enoxacin	Spain	69.3 – 323	López-Serna et al. (2013)
	Enrofloxacin	Spain	65.2 - 264	López-Serna et al. (2013)
	Erythromycin	Spain	41.3	López-Serna et al. (2013)
	Norfloxacin	Spain	81 - 462	López-Serna et al. (2013)
	Ofloxacin	Spain	43.3 - 367	López-Serna et al. (2013)
	Oxytetracycline	Spain	12.2 - 41	López-Serna et al. (2013)
	Roxithromycin	Spain	3.23 – 23.8	López-Serna et al. (2013)
		France	1.3	Vulliet & Cren-Olivé (2011)
	Flumequine	Spain	6.05 – 10.3	López-Serna et al. (2013)
	Josamycin	Spain	3.8	López-Serna et al. (2013)
	Spiramycin	Spain	17.2 - 2980	López-Serna et al. (2013)
	Sulfadiazine	Spain	37.1 - 208	López-Serna et al. (2013)
	Sulfamethazine	Spain	29.1 – 29.2	López-Serna et al. (2013)
	Sulfamethoxazole	Spain	18.2 – 65	López-Serna et al. (2013)

Antibiotics	Sulfamethoxazole	France	3.0	Vulliet & Cren-Olivé (2011)
	Tetracycline	Spain	56.3 – 141	López-Serna et al. (2013)
	Tilmicosin	Spain	5.71 – 820	López-Serna et al. (2013)
	Trimethoprim	Spain	4.89 – 9.41	López-Serna et al. (2013)
		France	1.4	Vulliet & Cren-Olivé (2011)
Nonsteroidal anti-inflammatory drug (NSAIDs)	Diclofenac	Spain	1.17 - 380	López-Serna et al. (2013)
		France	9.7	Vulliet & Cren-Olivé (2011)
	Ibuprofen	Serbia	92	Petrović et al. (2014)
		Spain	2.12 - 988	López-Serna et al. (2013)
	Naproxen	Serbia	27.6	Petrović et al. (2014)
		Spain	5.59	López-Serna et al. (2013)
		France	1.2	Vulliet & Cren-Olivé (2011)
	Phenazone	Serbia	23.4	Petrović et al. (2014)
		Spain	2.13 – 39.7	López-Serna et al. (2013)
	Ketoprofen	Spain	29.5 - 215	López-Serna et al. (2013)
		France	2.8	Vulliet & Cren-Olivé (2011)
	Mefenamic acid	Spain	13.5 – 64.3	López-Serna et al. (2013)
Antidepressants	Fluoxetine	Spain	21	López-Serna et al. (2013)
	Paroxetine	Spain	5.17 – 30.2	López-Serna et al. (2013)

Blood lipid lowering agents	Fenofibric acid	France	0.4	Vulliet & Cren-Olivé (2011)
	Atorvastatin	Spain	5.12 – 15.9	López-Serna et al. (2013)
	Bezafibrate	Spain	0.527 – 25.8	López-Serna et al. (2013)
	Fenofibrate	Spain	22.3 – 74.2	López-Serna et al. (2013)
	Gemfibrozil	Spain	0.821 - 751	López-Serna et al. (2013)
	Pravastatin	Spain	12.2	López-Serna et al. (2013)

Table S 3 - Pharmaceuticals concentration detected in seawater samples (expressed in ng L^{-1}).

Therapeutic Class	Pharmaceutical	Location	Concentration (ng L^{-1})	Reference
Antibiotics	Clarithromycin	Spain	17	Gros et al. (2012)
	Ofloxacin	Spain	2	Gros et al. (2012)
	Sulfamethoxazole	Spain	9	Gros et al. (2012)
	Trimethoprim	Spain	1	Gros et al. (2012)
Nonsteroidal anti-inflammatory drug (NSAIDs)	Diclofenac	Spain	4	Gros et al. (2012)
	Ibuprofen	Spain	16	Gros et al. (2012)
	Indomethacin	Spain	3	Gros et al. (2012)
	Naproxen	Spain	6	Gros et al. (2012)
	Phenazone	Spain	2	Gros et al. (2012)
Antidepressants	Citalopram	Spain	4	Gros et al. (2012)
	Venlafaxine	Spain	52	Gros et al. (2012)
	Trazadone	Spain	1	Gros et al. (2012)
Blood lipid lowering agents	Gemfibrozil	Spain	23	Gros et al. (2012)
	Atorvastatin	Spain	1	Gros et al. (2012)
	Bezafibrate	Spain	2	Gros et al. (2012)

Table S 4 - Pharmaceuticals concentration detected in drinking water samples (expressed in ng L^{-1}).

Therapeutic Class	Pharmaceutical	Location	Concentration (ng L^{-1})	Reference
Antibiotics	Sulfamethoxazole	Spain	0.5	Gros et al. (2012)
		Portugal	1.3	Gaffney et al. (2015)
		USA	12.7	Padhye et al. (2014)
		USA	1.3 – 8.2	Wang et al. (2011)
		USA	113	Schaider et al. (2014)
		Switzerland	15 - 17	Morasch (2013)
	Sulfadiazine	Portugal	1	Gaffney et al. (2015)
	Sulfamethazine	Portugal	0.5	Gaffney et al. (2015)
	Sulfapyridine	Portugal	1.9	Gaffney et al. (2015)
	Erythromycin	Portugal	5	Gaffney et al. (2015)
	Erythromycin-H_2O	USA	13.8	Padhye et al. (2014)
	Clarithromycin	USA	0.2	Padhye et al. (2014)
	Trimethoprim	USA	19.8	Padhye et al. (2014)
		USA	1.7 – 4.7	Wang et al. (2011)
		USA	0.7	Schaider et al. (2014)
		Switzerland	0.4 – 3	Morasch (2013)
	Sulfamethizole	USA	1	Schaider et al. (2014)
	Lincomycin	USA	2.0 – 4.4	Wang et al. (2011)
	Azithromycin	Switzerland	10	Morasch (2013)
	Norfloxacin	Switzerland	2	Morasch (2013)

Nonsteroidal anti-inflammatory drug (NSAIDs)	Ibuprofen	USA (raw)	5850	Loraine & Pettigrove (2006)
		USA (finished)	930	
		France	0.6	Togola & Budzinski (2008)
		Spain	5	Gros et al. (2012)
		Portugal	0.021	Gaffney et al. (2015)
		USA	10.2	Padhye et al. (2014)
		USA	2.0 – 72.8	Wang et al. (2011)
		Spain	21.24	Vazquez-Roig et al. (2010)
	Diclofenac	France	2.5	Togola & Budzinski (2008)
		Portugal	11	Gaffney et al. (2015)
		USA	9.4	Padhye et al. (2014)
		Switzerland	0.7 – 3	Morasch (2013)
	Ketoprofen	France	3.0	Togola & Budzinski (2008)
		Serbia	16	Petrović et al. (2014)
		Switzerland	4 – 8	Morasch (2013)
	Naproxen	France	0.2	Togola & Budzinski (2008)
		Portugal	6	Gaffney et al. (2015)
		USA	5.1	Padhye et al. (2014)
		Switzerland	4 – 12	Morasch (2013)
	Indomethacin	Spain	6	Gros et al. (2012)
		Portugal	37	Gaffney et al. (2015)

Nonsteroidal anti-inflammatory drug (NSAIDs)	Nimesulide	Portugal	27	Gaffney et al. (2015)
Antidepressants	Fluoxetine	USA	19.2	Padhye et al. (2014)
	Amitryptilline	France	1.4	Togola & Budzinski (2008)
Blood lipid lowering agents	Gemfibrozil	USA	1.2	Schaider et al. (2014)
		Portugal	18	Gaffney et al. (2015)
		Spain	8	Gros et al. (2012)
	Atorvastatin	Spain	1	Gros et al. (2012)

Table S 5 - Pharmaceuticals concentration detected in sediment and soils samples (expressed in ng g^{-1}).

Therapeutic Class	Pharmaceutical	Location	Concentration (ng g^{-1})	Reference
Antibiotics	Ciprofloxacin	Spain	4.6 – 7.3	Vazquez-Roig et al. (2012)
		Spain	5.95	Vazquez-Roig et al. (2010)
		Spain	0.10 – 3.79	Osorio et al. (2016)
		China	42	Wu et al. (2014)
	Norfloxacin	Spain	6.8 – 8.4	Vazquez-Roig et al. (2012)
		Malaysia	18 - 96	Ho et al. (2014)
		China	17.9	Wu et al. (2014)
	Ofloxacin	Spain	8.95 – 12.03	Vazquez-Roig et al. (2010)
		Spain	0.09 – 2.99	Osorio et al. (2016)
		Spain	2.7 - 3.3	Vazquez-Roig et al. (2012)
	Sulfamethoxazole	Spain	1.1	Vazquez-Roig et al. (2012)
		USA	0.7	Klosterhaus et al. (2013)
		Spain	0.07 – 0.26	Osorio et al. (2016)
		Poland	2.34 – 419.2	Siedlewicz et al. (2016)
	Tetracycline	Spain	6.5	Vazquez-Roig et al. (2012)
		Spain	5.92	Osorio et al. (2016)
	Trimethoprim	Spain	0.2 - 1.6	Vazquez-Roig et al. (2012)
		Spain	0.02-0.25	Biel-Maeso et al. (2017)
		USA	18.2	Klosterhaus et al. (2013)
		Spain	0.03 – 0.19	Osorio et al. (2016)

Antibiotics	Trimethoprim	Poland	1.74 – 2.46	Siedlewicz et al. (2016)
		Malaysia	3 - 60	Ho et al. (2014)
	Sulfamethoxypyridazine	Spain	0.11 – 0.37	Biel-Maeso et al. (2017)
	Sulfamethazine	Spain	0.24 – 1.15	Biel-Maeso et al. (2017)
		Poland	1.76	Siedlewicz et al. (2016)
	Erithromycin	Spain	1.13	Osorio et al. (2016)
	Erithromycin-H_2O	USA	3.4	Klosterhaus et al. (2013)
	Azithromycin	Spain	23.92	Osorio et al. (2016)
	Clarithromycin	Spain	12.72	Osorio et al. (2016)
	Cefalexin	Spain	0.40	Osorio et al. (2016)
	Metronidazole	Spain	0.12 – 12.61	Osorio et al. (2016)
	Sulfamethiazole	Poland	12.85 – 20.84	Siedlewicz et al. (2016)
	Doxycycline	Malaysia	63 - 728	Ho et al. (2014)
	Enrofloxacin	Malaysia	36 - 378	Ho et al. (2014)
		China	24.4	Wu et al. (2014)
		Brazil	26.69	Leal et al. (2012)
	Flumequine	Malaysia	8 - 1331	Ho et al. (2014)
	Tylosin	Malaysia	6 - 679	Ho et al. (2014)
	Lomefloxacin	China	11	Wu et al. (2014)
	Sulfachloropyridazine	Poland	0.47 – 1.07	Siedlewicz et al. (2016)
	Sulfathiazole	Poland	1.77	Siedlewicz et al. (2016)
	Chloramphenicol	Spain	0.17 – 2.10	Biel-Maeso et al. (2017)

Nonsteroidal anti-inflammatory drug (NSAIDs)	Diclofenac	Spain	0.81 – 15.39	Biel-Maeso et al. (2017)
		Spain	1.29 – 4.14	Osorio et al. (2016)
		Poland	2.1	Kumirska et al. (2015)
	Ibuprofen	Spain	0.91 – 24.93	Biel-Maeso et al. (2017)
		Spain	12.56	Osorio et al. (2016)
		Poland	1.0 – 8.0	Kumirska et al. (2015)
	Indomethacin	Spain	0.47 – 8.99	Biel-Maeso et al. (2017)
		Spain	0.47 – 2.94	Osorio et al. (2016)
	Phenazone	Spain	0..06	Osorio et al. (2016)
	Ketoprofen	Spain	5.79 - 12.54	Osorio et al. (2016)
	Naproxen	Spain	0.82 – 3.38	Osorio et al. (2016)
	Piroxicam	Spain	0.15	Osorio et al. (2016)
	Meloxicam	Spain	0.08	Osorio et al. (2016)
	Tenoxicam	Spain	0.66	Osorio et al. (2016)
	Flurbiprofen	Poland	6.5 – 8.8	Kumirska et al. (2015)
	Mefenamic acid	Spain	0.18 – 3.34	Biel-Maeso et al. (2017)
Antidepressants	Fluoxetine	Spain	0.34	Osorio et al. (2016)
	Norfluoxetine	Spain	0.14 – 0.60	Osorio et al. (2016)
	Paroxetine	Spain	0.05 – 0.76	Osorio et al. (2016)
	Sertraline	Spain	1.15– 119.28	Osorio et al. (2016)

Antidepressants	Citalopram	Spain	0.23 – 7.79	Osorio et al. (2016)
	Venlafaxine	Spain	0.05 – 1.94	Osorio et al. (2016)
	Trazodone	Spain	0.09 – 8.08	Osorio et al. (2016)
Blood lipid lowering agents	Bezafibrate	Spain	0.09 – 0.41	Osorio et al. (2016)
	Gemfibrozil	Spain	0.16 – 1.92	Osorio et al. (2016)
		Spain	0.08 – 0.39	Biel-Maeso et al. (2017)
	Atorvastatin	Spain	0.03-0.65	Osorio et al. (2016)
	Pravastatin	Spain	0.30	Osorio et al. (2016)
	Fluvastatin	Spain	0.22 – 4.53	Osorio et al. (2016)
	Fenofibrate	Spain	16.1	Vazquez-Roig et al. (2012)
		Spain	13.20 – 17.23	Vazquez-Roig et al. (2010)

Table S 6 - Pharmaceuticals concentration detected in sludge samples (expressed in ng g^{-1}).

Therapeutic Class	Pharmaceutical	Location	Concentration (ng g^{-1})	Reference
Antibiotics	Trimethoprim	UK	21.5	Petrie et al. (2016)
		Malaysia	9 - 3412	Ho et al. (2014)
	Doxycycline	Malaysia	309 – 78516	Ho et al. (2014)
		China	1050 – 10910	Zhao et al. (2010)
	Enrofloxacin	Malaysia	112 – 26863	Ho et al. (2014)
		Brazil	30970	Leal et al. (2012)
		China	33260 – 1420760	Zhao et al. (2010)
	Erythromycin	Malaysia	12 – 32	Ho et al. (2014)
	Norfloxacin	Malaysia	31 - 1886	Ho et al. (2014)
		Brazil	4550	Leal et al. (2012)
		China	2760 – 225450	Zhao et al. (2010)
	Sulfadiazine	Malaysia	12 – 5773	Ho et al. (2014)
		China	800 – 3120	Zhao et al. (2010)
	Tilmicosin	Malaysia	13 - 85	Ho et al. (2014)
	Tylosin	Malaysia	100 - 13740	Ho et al. (2014)
	Ciprofloxacin	Brazil	2130	Leal et al. (2012)
		China	29590 – 45590	Zhao et al. (2010)
	Fleroxacin	China	2220 – 99430	Zhao et al. (2010)
	Flumequine	Malaysia	21 - 51912	Ho et al. (2014)
	Sulfamethoxazole	China	840 – 2800	Zhao et al. (2010)

Antibiotics	Oxytetracycline	China	10560 – 59590	Zhao et al. (2010)
	Chlortetracycline	China	17680 – 27590	Zhao et al. (2010)
	Methacycline	China	960 – 5860	Zhao et al. (2010)
	Lomefloxacin	China	5530 – 44160	Zhao et al. (2010)
	Danofloxacin	China	2480 – 3060	Zhao et al. (2010)
	Sulfanilamide	China	40 – 1590	Zhao et al. (2010)
	Sulfamerazine	China	90 – 660	Zhao et al. (2010)
	Sulfadimidine	China	180 – 6040	Zhao et al. (2010)
	Difloxacin	China	2630 – 12380	Zhao et al. (2010)
	Sulfamonomethoxine	China	60 - 4080	Zhao et al. (2010)
	Sulfaguanidine	China	250 - 1550	Zhao et al. (2010)
	Sulfachloropyridazine	China	360 - 3510	Zhao et al. (2010)
Nonsteroidal anti-inflammatory drug (NSAIDs)	Ibuprofen	UK	174	Petrie et al. (2016)
		Poland	96	Kumirska et al. (2015)
	Diclofenac	UK	23.5	Petrie et al. (2016)
		Poland	20	Kumirska et al. (2015)
	Naproxen	UK	39.8	Petrie et al. (2016)
		Poland	10	Kumirska et al. (2015)
	Flurbiprofen	Poland	98	Kumirska et al. (2015)
Antidepressants	Citalopram	UK	657	Petrie et al. (2016)

Antidepressants	Venlafaxine	UK	37.9	Petrie et al. (2016)
	Fluoxetine	UK	188	Petrie et al. (2016)
	Sertraline	UK	1138	Petrie et al. (2016)
	Norfluoxetine	UK	124	Petrie et al. (2016)
	Mirtazapine	UK	66.1	Petrie et al. (2016)

Table S 7 - Pharmaceuticals concentration detected in wastewaters effluent samples (expressed in ng L^{-1}).

Therapeutic Class	Pharmaceutical	Location	Concentration (ng L^{-1})	Reference
Antibiotics	Erythromycin	UK	466 - 1857	Kay et al. (2017)
		Spain	82	Gracia-Lor et al. (2011)
		Spain	14 - 17	Gros et al. (2012)
		China	42 – 244.0	Yan et al. (2014)
	Ciprofloxacin	Spain	2292	Gracia-Lor et al. (2011)
		Spain	104 - 245	Gros et al. (2012)
		USA	2200	Blair et al. (2015)
		Serbia	278	Petrović et al. (2014)
		Spain	3194 - 4719	Mijangos et al. (2018)
	Clarithromycin	Spain	247	Gracia-Lor et al. (2011)
		Spain	19 - 192	Gros et al. (2012)
		UK	1065	Petrie et al. (2016)
		USA	8100	Blair et al. (2015)
	Enrofloxacin	Spain	220	Gracia-Lor et al. (2011)
		USA	34	Blair et al. (2015)
	Flumequine	Spain	41	Gracia-Lor et al. (2011)
		USA	15.6	Blair et al. (2015)
	Lincomycin	Spain	142	Gracia-Lor et al. (2011)
		USA	32	Blair et al. (2015)
	Nalidixic acid	Spain	60	Gracia-Lor et al. (2011)

Antibiotics	Moxifloxacin	Spain	540	Gracia-Lor et al. (2011)
		China	5.1 – 7.7	Yan et al. (2014)
	Azithromycin	Spain	31 - 170	Gros et al. (2012)
		UK	87.2	Petrie et al. (2016)
		China	20.7 – 446.5	Yan et al. (2014)
		USA	1300	Blair et al. (2015)
	Ofloxacin	Spain	157 - 191	Gros et al. (2012)
		China	26.7 – 310.0	Yan et al. (2014)
		USA	2100	Blair et al. (2015)
		Serbia	220	Petrović et al. (2014)
		Spain	925	Gracia-Lor et al. (2011)
	Sulfamethoxazole	Spain	222	Gros et al. (2012)
		UK	47.5	Petrie et al. (2016)
		China	44.5 - 1296.3	Yan et al. (2014)
		USA	7400	Blair et al. (2015)
		Serbia	432	Petrović et al. (2014)
		Spain	432	Gracia-Lor et al. (2011)
		Spain	190 - 8963	Mijangos et al. (2018)
		Mexico	440 - 1215	Rivera-Jaimes et al. (2018)
	Trimethoprim	Spain	10 – 100	Gros et al. (2012)
		UK	769	Petrie et al. (2016)
		China	6.7 – 188.0	Yan et al. (2014)

Antibiotics	Trimethoprim	USA	570	Blair et al. (2015)
		Serbia	259	Petrović et al. (2014)
		Spain	232	Gracia-Lor et al. (2011)
		Spain	61 - 5843	Mijangos et al. (2018)
		Mexico	130 - 395	Rivera-Jaimes et al. (2018)
	Sulfamerazine	USA	30	Blair et al. (2015)
	Sulfamethizole	USA	60	Blair et al. (2015)
	Cefalexin	Serbia	803	Petrović et al. (2014)
	Ampicillin	USA	160	Blair et al. (2015)
	Penicillin G	USA	30	Blair et al. (2015)
	Penicillin V	USA	86	Blair et al. (2015)
	Sarafloxacin	USA	130	Blair et al. (2015)
		Spain	52	Gracia-Lor et al. (2011)
	Sulfadiazine	China	14 - 189.7	Yan et al. (2014)
		USA	20	Blair et al. (2015)
		Spain	24 - 5477	Mijangos et al. (2018)
	Sulfamethazine	China	2 - 48.6	Yan et al. (2014)
		USA	40	Blair et al. (2015)
	Sulfamethazine	Spain	11	Gracia-Lor et al. (2011)
	Sulfadimethoxine	USA	52	Blair et al. (2015)
	Sulfachloropyridazine	USA	30	Blair et al. (2015)
	Pefloxacin	Spain	122	Gracia-Lor et al. (2011)

Antibiotics	Norfloxacin	China	9.4 - 134.3	Yan et al. (2014)
		USA	140	Blair et al. (2015)
		Spain	310	Gracia-Lor et al. (2011)
		Spain	40 - 463	Mijangos et al. (2018)
	Roxithromycin	China	36.5 – 512.4	Yan et al. (2014)
		USA	120	Blair et al. (2015)
		Spain	18	Gracia-Lor et al. (2011)
	Sulfathiazole	USA	92	Blair et al. (2015)
		Spain	30	Gracia-Lor et al. (2011)
	Pipedimic acid	Spain	430	Gracia-Lor et al. (2011)
	Mefenamic acid	UK	15 - 108	Kay et al. (2017)
	Metronidazole	Spain	121	Gros et al. (2012)
Nonsteroidal anti-inflammatory drug (NSAIDs)	Diclofenac	France	210.7–486.4	Togola & Budzinski (2008)
		UK	401 - 2830	Kay et al. (2017)
		Spain	690	Gracia-Lor et al. (2011)
		Spain	184 – 376	Gros et al. (2012)
	Diclofenac	UK	436	Petrie et al. (2016)
		China	1.0 - 4.7	Yan et al. (2014)
		Serbia	1338	Petrović et al. (2014)
		Mexico	466 - 2180	Rivera-Jaimes et al. (2018)
		Spain	683 - 1932	Mijangos et al. (2018)

Nonsteroidal anti-inflammatory drug (NSAIDs)	Ibuprofen	France	17.7–219.0	Togola & Budzinski (2008)
		UK	863 - 4617	Kay et al. (2017)
		Spain	15100	Gracia-Lor et al. (2011)
		UK	1290	Petrie et al. (2016)
		China	3.5 – 41.6.	Yan et al. (2014)
		USA	4500	Blair et al. (2015)
		Serbia	20130	Petrović et al. (2014)
	Ketoprofen	France	21.8–1080.6	Togola & Budzinski (2008)
		Spain	583	Gracia-Lor et al. (2011)
		Spain	39 – 560	Gros et al. (2012)
		Serbia	247	Petrović et al. (2014)
		Spain	13 - 374	Mijangos et al. (2018)
	Naproxen	France	42.1–289.1	Togola & Budzinski (2008)
		Spain	710	Gracia-Lor et al. (2011)
		Spain	97 – 150	Gros et al. (2012)
		UK	3516	Petrie et al. (2016)
	Naproxen	USA	3000	Blair et al. (2015)
		Serbia	208	Petrović et al. (2014)
		China	6.7 - 7.7	Yan et al. (2014)
		Mexico	49 - 392	Rivera-Jaimes et al. (2018)
	Phenazone	Spain	9 – 49	Gros et al. (2012)
		Serbia	13.5	Petrović et al. (2014)

Nonsteroidal anti-inflammatory drug (NSAIDs)	Piroxicam	Spain	87	Gros et al. (2012)
	Meloxicam	Spain	735	Gros et al. (2012)
		Serbia	5.0	Petrović et al. (2014)
	Tenoxicam	Spain	19	Gros et al. (2012)
	Indomethacin	Mexico	38 - 305	Rivera-Jaimes et al. (2018)
Antidepressants	Citalopram	Spain	49 - 288	Gros et al. (2012)
		UK	323	Petrie et al. (2016)
		China	2 – 162	Yuan et al. (2013)
	Venlafaxine	Spain	364 - 376	Gros et al. (2012)
		UK	355	Petrie et al. (2016)
		Serbia	154	Petrović et al. (2014)
		Spain	875	Gracia-Lor et al. (2011)
	Trazodone	Spain	29	Gros et al. (2012)
	Amitryptiline	France	6.0	Togola & Budzinski (2008)
	Fluoxetine	Spain	28	Gros et al. (2012)
		UK	26.5	Petrie et al. (2016)
		China	10	Yuan et al. (2013)
		USA	50	Blair et al. (2015)
	Paroxetine	Spain	386	Gros et al. (2012)
	Sertraline	UK	47	Petrie et al. (2016)
		China	9 – 59	Yuan et al. (2013)

Antidepressants	Mirtazapine	UK	55	Petrie et al. (2016)
	Chlorimipramine	China	4-35	Yuan et al. (2013)
	Norfluoxetine	UK	30	Petrie et al. (2016)
Blood lipid lowering agents	Gemfibrozil	France	13.3–17.2	Togola & Budzinski (2008)
		USA	190	Blair et al. (2015)
		China	0.6 – 10.2	Yan et al. (2014)
		Spain	2008	Gracia-Lor et al. (2011)
		Spain	178 - 1018	Gros et al. (2012)
		Mexico	20 - 380	Rivera-Jaimes et al. (2018)
	Bezafibrate	China	2.7 – 128.1	Yan et al. (2014)
		UK	892	Petrie et al. (2016)
		Spain	312	Gracia-Lor et al. (2011)
		Spain	7 - 26	Gros et al. (2012)
		Spain	40 - 132	Mijangos et al. (2018)
		Mexico	265 - 950	Rivera-Jaimes et al. (2018)
	Atorvastatin	China	0.7 – 0.8	Yan et al. (2014)
		UK	60.5	Petrie et al. (2016)
		Spain	209	Gracia-Lor et al. (2011)
		Spain	27 - 111	Gros et al. (2012)
		Serbia	40.5	Petrović et al. (2014)
		Canada	10 - 122	Lee et al. (2009)

Blood lipid lowering agents	Pravastatin	Spain	69	Gracia-Lor et al. (2011)
		Spain	36	Gros et al. (2012)
	Fluvastatin	Spain	12	Gros et al. (2012)
	Simvastatin	China	8.4 - 129	Yan et al. (2014)
	Rosuvastatin	Canada	190 - 552	Lee et al., 2009

Appendix II

Chapter 2

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SM 1 - Mineral-salts medium (MM) constitution

The Mineral-salts medium (MM) contains the following composition **(per litre of deionized water):**

- 2.7 g of di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot {}_2H_2O$)
- 1.4 g of Potassium dihydrogen phosphate (KH_2PO_4)
- 0.5 g of Ammonium sulfate ($(NH_4)_2SO_4$)
- 0.2 g of Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)
- 10 mL of trace elements solution with the following composition, per litre:
    - 12.0 g of Disodium ethylenediaminetetraacetate dihydrate ($Na_2EDTA \cdot 2H_2O$)
    - 2.0 g of Sodium hydroxide (NaOH)
    - 0.4 g of Manganese (II) Sulfate Tetrahydrate ($MnSO_4 \cdot 4H_2O$)
    - 0.4 g of Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)
    - 0.5 mL of concentrated Sulfuric acid (H_2SO_4)
    - 10.0 g of Sodium sulfate (Na_2SO_4)
    - 0.1 g Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$)
    - 2.0 g of Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)
    - 0.1 g of Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$)
    - 1.0 g of Calcium chloride ($CaCl_2$)